(11) **EP 1 193 314 B1**(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention  
of the grant of the patent:  
20.10.2004 Bulletin 2004/43

(51) Int Cl.7: C12N 9/20, C07K 19/00,  
C12N 15/80, A21D 8/04

(21) Application number: 01204340.2

(22) Date of filing: 03.04.1998

(54) Use of lipase for improving doughs and baked products

Verwendung von Lipase zur Verbesserung von Teigen und Backwaren

Utilisation de lipase pour améliorer des pâtes à pain et des produits de boulangerie

(84) Designated Contracting States:  
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU  
MC NL PT SE

(74) Representative: Harding, Charles Thomas  
D. Young & Co.  
21 New Fetter Lane  
London EC4A 1DA (GB)

(30) Priority: 09.04.1997 DK 40097

(43) Date of publication of application:  
03.04.2002 Bulletin 2002/14

(56) References cited:  
EP-A- 0 575 133 EP-A- 0 585 988  
EP-A- 0 808 803 WO-A-94/04035  
US-A- 5 232 846

(83) Declaration under Rule 28(4) EPC (expert  
solution)

(62) Document number(s) of the earlier application(s) in  
accordance with Art. 76 EPC:  
98913539.7 / 0 977 869

(73) Proprietor: DANISCO A/S  
1001 Copenhagen K. (DK)

(72) Inventors:  
• Poulsen, Charlotte Horsmans  
8220 Braband (DK)  
• Soe, Jom Borch  
8381 Mundelstrup (DK)  
• Rasmussen, Preben  
4070 Kirke Hyllinge (DK)  
• Madrid, Susan Mampusta  
2960 Vedbaek (DK)  
• Zargahl, Masoud R.  
8239 Abyhøj (DK)

- DIEGO COLOMBO ET AL: "Optically pure 1-O- and 3-O-Beta-D-Glucosyl- and Galactosyl-sn-glycerols through lipase-catalyzed transformations " TETRAHEDRON LETTERS, vol. 36, no. 27, 1995, pages 4865-4868, XP004027788
- BILYK A: "LIPASE-CATALYZED TRIGLYCERIDE HYDROLYSIS IN ORGANIC SOLVENT1" JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, AMERICAN OIL CHEMISTS' SOCIETY, CHAMPAIGN, US, vol. 68, no. 5, 1 May 1991 (1991-05-01), pages 320-323, XP000205336 ISSN: 0003-021X
- ATSUSHI TSUCHIYA ET AL: "Cloning and nucleotide sequence of the mono- and diacylglycerol lipase gene (mdlB) of Aspergillus oryzae" FEMS MICROBIOLOGY LETTERS, vol. 143, 1996, pages 63-67, XP001063364

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

EP 1 193 314 B1

BEST AVAILABLE COPY

## EP 1 193 314 B1

## Description

## FIELD OF THE INVENTION

5 [0001] The present invention relates to the field of food manufacturing, in particular to the preparation of improved bakery products. Specifically, the invention provides the use of a polypeptide having lipase activity which is capable of conferring improved characteristics to food products including bakery products.

## BACKGROUND OF THE INVENTION AND PRIOR ART

10 [0002] Lipases (EC 3.1.1.3), which can be defined as carboxylesterases which catalyze the hydrolysis of acylglycerols, are physiologically very important enzymes as one of the three major digestive enzymes together with amylases and proteases. They hydrolyse lipids to glycerol and fatty acids, but can also function in esterification or transesterification reactions.

15 [0003] Several studies report on the purification and characterisation of lipases from *Aspergillus niger*. Thus, Tombs and Blake (Biochim. Biophys., 1982, 700:81-89) purified a lipase from a commercial crude *Aspergillus* medium concentrate. The pure lipase was a glycosylated dimer containing two chains each having a molecular weight of 25 kDa.

[0004] Iwai and Tsujisaka (in Lipases, Borgström and Brockman (eds.), Elsevier, Amsterdam, 1984, pp 443-468) also purified an extracellularly secreted lipase from *Aspergillus niger* and obtained crystals of the lipase. They determined the molecular weight of the lipase to be 38 kDa and found that the enzyme was monomeric. The pI was determined to be 4.3. Optimum pH on olive oil was 5.6 and the optimum temperature on the same substrate was 25°C. The lipase was stable in a pH range of from 2.2 to 6.8 (30°C, 24 hours) and up to a temperature of 50°C (pH 5.6, 15 min). The lipase displayed a high activity towards triglycerides of medium chain length fatty acids.

20 [0005] Höfelmann et al. (J. Food Sci., 1985, 50:1721-1731) used a commercial *Aspergillus niger* lipase product (Lipase 2212, Röhm) as starting material for purification of lipase. Two lipases with molecular weight 19 kDa and 31 kDa and a pI of 3.5 and 4.0, respectively were obtained. Both enzymes were glycosylated.

[0006] Torossian and Bell (Biotechnol. Appl. Biochem., 1991, 13:205-211) used a crude commercial lipase preparation from *Aspergillus niger* from Amano (Japan). They determined the molecular weight to be 37 kDa and pI was 4.0. The N-terminal was determined to be XVSTSTLDELQFALQ. Sugihara et al. (Agric. Biol. Chem., 1988, 52:1591-1592) found the N-terminal to be SVT and the molecular weight to be 35 kDa for a lipase purified from an Amano (Japan) *Aspergillus niger* lipase preparation.

[0007] Despite the discrepancies in molecular weight for the mentioned lipases they were all reported to be 1,3-specific with regard to the hydrolysis of triglycerides.

35 [0008] Within the baking industry it is well known to use enzymes, such as amylases, xylanases, oxidases and proteases, for the improvement of the dough, the dough handling properties and/or the baked product to obtain increased volume, retarded staling and greater softness. The use of lipases as baking additive is also known.

[0009] Thus, US 3,368,903 discloses purified lipase preparations isolated from plant seeds which, when added to a bread dough mixture, has a significant bread staling retarding effect.

40 [0010] JP-62-285749-A describes a method of bread making in which lipase is added to the dough in admixture with vital gluten and lecithin. It is stated that this lipase deteriorates quality properties such as bread volume and elasticity of the crumb.

[0011] Mohsen et al. (Egypt. J. Food Sci., 1986, 14:175-182) describes that a lipase produced by *Rhizopus delemar* improves the softness of bread.

45 [0012] A bread improver comprising glucose oxidase in combination with oxidases other than glucose oxidase or hydrolases such as for example lipase is disclosed in EP 468 731 A1. There is obtained bread of a sufficient volume which is satisfactory in the quality of the internal and external characteristics. However, the use of lipase alone has a bread volume effect.

[0013] WO-94/04035 discloses a method of improving the properties of a dough (with and without added fat) and/or a baked product made from the dough by adding a lipase of microbial origin to the dough. The use of the microbial lipase resulted in an increased volume and improved softness of the baked product. Furthermore, an improved anti-staling effect was found.

50 [0014] EP 585 988 A1 discloses a bread improver composition comprising at least one lipase, at least one hemicellulase and at least one amylase. Baking experiments showed that the use of lipase alone in a dough without added fat resulted in a reduced volume of the baked product whereas no volume effect was observed when lipase is used in a dough containing added fat.

55 [0015] From the prior art it can thus be derived that the effects of known lipases when used as dough additives are highly variable in respect of antistaling or crumb firmness retardation and bread volume.

[0016] The present invention provides the use of a polypeptide having lipase activity in the preparation of a dough

## EP 1 193 314 B1

and/or baked product and which polypeptide is a triacylglycerol hydrolysing enzyme and wherein said polypeptide is capable of splitting off fatty acids having short, medium and long chain length, characterised in that said polypeptide is capable of hydrolysing galactolipids that are normally present in the flour to the corresponding galactosyl monoglycerides and wherein said polypeptide is capable of hydrolysing at least 10% of the galactosyl diglycerides normally present in the flour dough to galactosyl monoglycerides and wherein the enzyme imparts one or more of the following characteristics on the baked product improved pore homogeneity and reduced pore diameter.

[0017] Novel polypeptides having lipase activity which were found to confer highly desirable characteristics not disclosed in the prior art to doughs and bakery products may be used in accordance with the present invention. Thus, in baking experiments these polypeptides showed surprising and not previously taught or suggested properties when used in flour doughs, including increased crumb pore homogeneity and reduced pore diameter without concomitant negative effects on bread volume and crumb porosity. Thus, the use according to the invention provides baked products being less prone to mechanical deformation.

[0018] Small average pore diameter, increased pore homogeneity and unchanged porosity imply that the invention provides the means of obtaining baked products with a reinforced crumb structure. The improved pore homogeneity of the baked product further implies the advantage that there is obtained a product which is more sliceable and resistant to physical handling due to the reinforced crumb structure.

[0019] It is well known that it is difficult to spread thin layers of butter or margarine onto slices of bread having a very inhomogeneous pore structure. Therefore, it is an advantage that bread, as can be obtained in accordance with the present invention, has a fine and homogeneous pore structure.

[0020] It is well-known that a sliced loaf is less resistant to physical handling than un-sliced loaf. Therefore, the reinforced crumb structure, which is obtained by adding the polypeptide of the present invention to the dough, is particularly advantageous in baked products, such as toast bread, which are typically sliced immediately after baking by the manufacturer and are distributed in sliced condition to fast-food shops and supermarkets.

[0021] It has further been found that the use according to the present invention improves the stability of the gluten network in flour doughs which implies the advantage that the tolerance to variations in fermentation time is enhanced.

[0022] It is therefore an important objective of the present invention to provide such useful lipase active polypeptides. It has been found that such polypeptides may be derived from filamentous fungi such as e.g. *Aspergillus tubigenis*. However, the polypeptide is only produced in small amounts in wild-type fungal strains. Another important objective is therefore to provide a method of producing the novel polypeptides in a cost-effective manner by using recombinant DNA technology.

## SUMMARY OF THE INVENTION

[0023] Accordingly, the present invention relates to the use of a polypeptide having lipase activity in the preparation of a dough and/or baked product and which polypeptide is a triacylglycerol hydrolysing enzyme and wherein said polypeptide is capable of splitting off fatty acids having short, medium and long chain length, characterised in that said polypeptide is capable of hydrolysing galactolipids that are normally present in the flour to the corresponding galactosyl monoglycerides and wherein said polypeptide is capable of hydrolysing at least 10% of the galactosyl diglycerides normally present in the flour dough to galactosyl monoglycerides, wherein said polypeptide retains at least 80% activity after 4 days at 20°C and at a pH in the range of 3.5-8; and wherein the enzymes imparts one or more of the following characteristics on the baked product improved pore homogeneity and reduced pore diameter.

[0024] A polypeptide for use in accordance with the present invention having lipase activity that is derivable from *Aspergillus tubigenis*, the polypeptide having the following characteristics: (i) it retains at least 80% activity after 4 days at 20°C at a pH in the range of 3.5-8, (ii) it retains at least 60% of its activity after 1 hour at 60°C in 100 mM sodium acetate buffer at pH 5.0, and (iii) it has an isoelectric point as determined by isoelectric focusing in the range of 3.5-4.5. Specifically, the polypeptide is one that comprises at least one amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3, where Xaa in said sequences is an amino acid selected from the group consisting of Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

[0025] A recombinant DNA molecule comprising a nucleotide sequence coding for the above lipase active polypeptide is also taught herein.

[0026] A cell comprising the recombinant DNA molecule that is capable of expressing the polypeptide having lipase activity is also referred to herein by way of reference.

[0027] There is also taught herein a method of preparing the polypeptide for use in accordance with the invention, the method comprising transforming a host cell with a recombinant DNA molecule comprising a sequence coding for the polypeptide, the host cell is capable of expressing the nucleotide sequence coding for the polypeptide, cultivating the transformed host cell under conditions where the nucleotide sequence is expressed and harvesting the polypeptide.

[0028] A method of preparing a baked product having improved pore homogeneity and reduced pore diameter, the

## EP 1 193 314 B1

method comprising adding the polypeptide in accordance with the use of the invention to the dough.

[0029] Thus, the present invention relates to the use of the polypeptide having lipase activity in a dough for a baked product to improve the stability of the gluten network in the dough or to impart improved pore homogeneity or reduced pore diameter to the baked product and a dough improving composition comprising the polypeptide and at least one further conventional dough additive component.

## DETAILED DISCLOSURE OF THE INVENTION

[0030] As it is mentioned above, the invention relates to the use of a polypeptide having lipase activity. As used herein the term "lipase" is used to designate any triacylglycerol hydrolysing enzyme, including such enzymes that are capable of splitting off fatty acids having short, medium and long chain length. According to the invention, the lipase active polypeptide is one which retains at least 80% activity after 4 days at 20°C and at a pH in the range of 3.5-8 including a pH in the range of 5-7.

[0031] To be practically useful, it is also advantageous that the polypeptide for use in accordance with the invention has a good thermotolerance and optimum temperature for activity, at least to an extent where it is fully active in a dough at least up to the proofed dough is heated in an oven. Preferably, the lipase has a thermostability which renders the enzyme active during at least part of the baking process. Specifically, the thermostability of the polypeptide is at a level where it retains at least 60% of its activity after 1 hour at 60°C in 100 mM sodium acetate buffer at pH 5.0, including a polypeptide that retains at least 80% of its activity after 1 hour at 50°C under the same conditions.

[0032] In one specific embodiment, the polypeptide for use in accordance with the invention comprises at least one amino acid sequence shown herein as SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3.

[0033] In advantageous embodiments, the polypeptide for use in accordance with the invention shows enzymatic activity at a pH in the range of 3.5-8.0, including the range of 5-7.

[0034] The polypeptide for use in accordance with the present invention has an isoelectric point determined by isoelectric focusing in the range of 3.5-4.5 such as the range of 3.8 to 4.2 including an isoelectric point of 4.0±0.1.

[0035] A highly advantageous characteristic of the polypeptide for use in accordance with the invention is its capability to hydrolyse galactolipids such as galactosyl diglycerides, including digalactosyl diglyceride and monogalactosyl diglyceride, that are normally present in a flour, to the corresponding galactosyl monoglycerides. Thus, it has been found that the present polypeptides are capable of hydrolysing at least 10% of the galactosyl diglycerides normally present in a flour dough to monoglycerides. Preferably, at least 15% of these diglycerides, such as at least 25%, are hydrolysed.

[0036] The polypeptide for use in accordance with the invention can be in a glycosylated form. However, it has been found that the enzymatic activity of such a glycosylated lipase may be enhanced by deglycosylation. Accordingly, it may be preferred to provide the polypeptide in a non-glycosylated form. Thus, when the polypeptide is obtained from its natural source or from a recombinant host cell in a glycosylated form, its activity can be enhanced by N-deglycosylation by at least partially removing carbohydrate moieties by digestion with a deglycosylating enzyme such as endo-β-N-acetyl-glucosamidase H.

[0037] Alternatively, a non-glycosylated polypeptide for use in accordance with the invention is provided by means of modifying a DNA sequence coding for such polypeptide so as to provide a coding sequence that does not code for amino acids or a subsequence of the polypeptide that provides glycosylation sites. As it will be explained in the following, such mutated sequences coding for mutant polypeptides having lipase activity can be provided which, relative to the wild-type polypeptide, have an enhanced enzymatic activity.

[0038] The degree of glycosylation of the polypeptide as obtained is reflected in the molecular weight. As an example, when the polypeptide for use in accordance with the invention is derived from *Aspergillus tubigensis* in a glycosylated form, it typically has a molecular weight as determined by gel filtration using Superose 12 of 32±1 kDa. By matrix-assisted laser desorption ionisation mass spectrometry (MALDI-MS) the polypeptide for use in accordance with the invention typically has a molecular weight of 31±1.5 kDa. It has been found that in this initially glycosylated polypeptide, N-linked oligosaccharides account for about 10% of the polypeptide.

[0039] In one specific embodiment, the polypeptide for use in accordance with the invention comprises the amino acid sequence shown herein as SEQ ID NO:9 or a variant, homologue or fragment hereof.

[0040] In the present context, the terms "variant" or "fragment" in relation to the polypeptide for use in accordance with the present invention include any substitution, variation, modification, replacement, deletion or addition of one or more amino acids from or to the SEQ ID NO:9 sequence provided the resultant amino acid sequence has lipase activity, preferably at least the same activity as the polypeptide shown as SEQ ID NO:9.

[0041] In particular, the term "homologue" is used herein to include polypeptides having lipase activity which, relative to the sequence shown as SEQ ID NO:9, is of a similar amino acid composition or sequence, allowing for minor variations which do not have an adverse effect on the enzymatic properties and/or biological function, or which may give interesting and useful novel properties or biological functions. The homologous polypeptide may be derived from any organisms or it may also be derived through the use of recombinant DNA techniques whereby a naturally occurring



## EP 1 193 314 B1

polypeptide is modified in its sequence. Preferably, a homologous polypeptide is one where there is at least 75%, more preferably at least 85% and most preferably at least 95% homology to the sequence shown as SEQ ID No. 9.

[0042] In another embodiment of the invention, the polypeptide is part of a fusion protein that comprises further enzymatically active sequences. Although it is possible to construct such chimeric polypeptides by post-translational modifications, it is generally preferred to provide such fusion proteins by recombinant DNA means where a host cell is transformed with a DNA sequence coding for the fusion product and having this product expressed as a chimeric protein. Examples of such additional enzymatic activity include proteolytic, amylolytic and hemicellulolytic activities.

[0043] When a polypeptide is obtained from a cell expressing the gene coding for the polypeptide it is generally in the form of a more or less crude preparation containing other (contaminating) enzymatic activities. In accordance with the invention, it is also possible to provide the polypeptide in a substantially pure form. Such a purified polypeptide can be obtained by subjecting a crude enzyme preparation to any conventional method for purifying polypeptides and proteins.

[0044] According to the invention, the polypeptide is obtainable from *Aspergillus tubigensis* as it is described in the following examples. However, it can be derived from any organism that produces such a polypeptide, including fungi, yeast species, Gram-negative and Gram-positive bacteria, plant cells or animal cells, including human cells.

[0045] As it is mentioned above, interesting properties of the polypeptide for use in accordance with the invention is its capability to reduce the crumb pore diameter and to increase the pore homogeneity of the crumb of bread. In one specific embodiment, the polypeptide is one which, when it is added to a bread dough in an amount of 5,000 lipase units (LUS) per kg flour, reduces the average pore diameter of the crumb of the bread made from the dough by at least 10%, relative to a bread which is made from a bread dough without addition of the lipase. In another embodiment, the polypeptide is a polypeptide which when it is added to a bread dough in an amount of 5,000 LUS per kg flour, increases the pore homogeneity of the crumb of the bread made from the dough by at least 5%, relative to a bread which is made from a bread dough without addition of the lipase.

[0046] There is described herein for reference a recombinant DNA molecule comprising a nucleotide sequence coding for the polypeptide having lipase activity as it is described above.

[0047] Such a nucleotide sequence can be isolated from a natural source or it can be constructed as it is described in details in the below examples where such a coding sequence isolated from *Aspergillus tubigensis* and referred to as lipA is described in details. The nucleotide sequence can also be synthesised based on amino acid sequences of a naturally occurring polypeptide exhibiting lipase activity.

[0048] In useful embodiments, the recombinant DNA molecule comprises a nucleotide sequence coding for a polypeptide exhibiting lipase activity which comprises at least one of the amino acid sequences shown herein as SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3 or a nucleotide sequence coding for a polypeptide exhibiting lipase activity which comprises the amino acid sequence shown as SEQ ID No. 9.

[0049] In further specific embodiments, the recombinant DNA molecule comprises at least one of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:7 or at least the coding sequence of the nucleotide sequence shown as SEQ ID NO:8 or a variant, homologue or fragment thereof, or a sequence complementary thereto.

[0050] In the present context, the terms "variant" or "fragment" in relation to the nucleotide sequence coding for the polypeptide of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition of one or more nucleic acids from or to the sequence providing the resultant nucleotide sequence coding for a polypeptide having lipase activity, preferably having at least the same activity as the polypeptide shown as SEQ ID NO:9.

[0051] The term "homologue" covers homology with respect to sequence and/or structure and/or function providing the resultant nucleotide sequence codes for a polypeptide having lipase activity. With respect to sequence homology (i.e. similarity), preferably there is at least 75%, more preferably at least 85%, most preferably at least 95% homology to the sequence shown as SEQ ID NO:8.

[0052] The recombinant DNA molecule may advantageously comprise a sequence that codes for a polypeptide according to invention which does not comprise amino acid(s) providing glycosylation site(s). Such useful recombinant DNA molecules can be selected from the plasmids deposited under the accession Nos. NCIMB 40931, NCIMB 40932, NCIMB 40933, NCIMB 40934 and NCIMB 40935.

[0053] A cell comprising a recombinant DNA molecule as described above and which is capable of expressing the polypeptide for use in accordance with the invention is also taught herein. Such a cell can be selected from fungi, yeast species, bacteria, plant cells and animal cells including human cells. Useful cells are selected from filamentous fungi such as an *Aspergillus* sp., a *Penicillium* sp., a *Rhizomucor* sp., a *Mucor* sp., a *Trichoderma* sp. including *T. reesei*, *T. viridae* and *T. longibrachiatum*, a *Neurospora* sp. and a *Humicola* sp. Suitable *Aspergillus* species include *A. niger*, *A. tubigensis*, *A. oryzae* and *A. awamori*.

[0054] Useful bacterial host cells include Gram-negative species such as e.g. *E. coli* including the *E. coli* strain harbouring plasmid pLIP4 as deposited under the accession No. NCIMB 40863, and Gram-negative species such as e.g. *Bacillus* species and lactic acid bacterial species such as *Lactococcus lactis*.

## EP 1 193 314 B1

[0055] A method of preparing the polypeptide for use in accordance with the invention by expression of a nucleotide sequence capable of expressing the polypeptide in an appropriate transformed host cell is taught herein. The method comprises in a first step that a suitable transformable cell is transformed with the above recombinant DNA molecule to provide a transformed host cell expressing the polypeptide. Procedures for transformation of prokaryotic host cells is well documented in the art, for example see Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press).

[0056] The term "transformed host cell" is used herein to include any transformable organism wherein the nucleotide sequence coding for the enzyme according to the present invention has been introduced. The introduction of the coding sequence can be in the form of introducing an episomal replicon such as a plasmid, a bacteriophage or a cosmid into the cell. Such replicons can advantageously be introduced in multiple copies to obtain an increased expression of the polypeptide. It may in certain cases be advantageous that the nucleotide sequence is incorporated into the genome of the host cell organism e.g. by means of a transposable element or a recombinational event.

[0057] A presently preferred host organism for the expression of the nucleotide sequence of the present invention and/or for the preparation of the polypeptide of the present invention is an organism of the genus *Aspergillus*, such as *Aspergillus niger* or *Aspergillus tubigensis*.

[0058] A transformed *Aspergillus* strain according to the present invention can e.g. be prepared according to methods described by Rambosek and Leach (CRC Crit. Rev. Biotechnol., 1987, 6:357-393), Davis (in: Progress in Industrial Microbiology, Martinelli and Kinghorn (eds.), Elsevier Amsterdam, 1994, 29:525-560), Ballance (in: Molecular Industrial Mycology, Systems and Applications for Filamentous Fungi, Leong and Berka (eds.), Marcel Dekker Inc., New York 1991, pp 1-29) and Turner (in: Progress in Industrial Microbiology, Martinelli and Kinghorn (eds.), Elsevier Amsterdam, 1994, 29:641-666).

[0059] In another embodiment, the method makes use of a yeast host cell such as *Saccharomyces cerevisiae* or *Pichia pastoris*. Expression of heterologous genes in *Saccharomyces cerevisiae* has been reviewed by Goody et al. (in: Yeast Biotechnology, Berry et al. (eds.), Allen and Unwin, London, 1987, pp 401-429) and by King et al. (in: Molecular and Cell Biology of Yeasts, Walton and Yarrington (eds.), Blackie, Glasgow, 1989, pp 107-133).

[0060] Furthermore, the present invention relates in a particular aspect to the use of the polypeptide according to the invention to improve the stability of the gluten network in a flour dough and to impart improved pore homogeneity and reduced pore diameter to the baked product made from said dough. It has been found that the polypeptide has these improving effects in a fat-free dough.

[0061] In the present context, the term "fat-free dough" is used to indicate that no lipid or fat is added to the flour dough. A preferred flour is wheat flour or a composite flour wherein part of the wheat flour is replaced by starch, optionally supplemented with plant protein and additives such as emulsifiers. Other types of flour derived from rice, maize, barley and rye are also contemplated.

[0062] Thus, the major ingredients of the dough include flour, preferably wheat flour, water and an "gas generating substance" such as yeast or a chemical leavening agent. In addition to the above-mentioned major ingredients the dough may include minor ingredients such as salt, sugar, minerals, vitamins, flavouring and at least one further dough additive such as for example an emulsifying agent, a hydrocolloid, a starch degrading enzyme or a cellulose or hemicellulose degrading enzyme.

[0063] During mixing and moulding of the dough ingredients to provide a homogeneous dough the interaction between wheat gluten, starch and water is essential for obtaining a dough with good dough handling properties and a satisfactory baked product made from the dough.

[0064] It is generally assumed that starch and gluten form a structural network including the glycolipids in the form of liquid-crystalline phases of lamellar structure as layers between gluten protein and starch.

[0065] The crumb structure of a baked product can be evaluated by visual inspection of the bread cross section. However, a more reliable method giving a quantitative measure of the crumb structure is by image analysis using an image analyzer which on the entire cross section of bread separates and analyses the individual pores one by one and calculate the mean pore size diameter. The bread cross section in its entirety is characterised by the distribution of the individual pores, for example in the form of a histogram. By homogeneity is understood the uniformity of the pore size and in the present context the term "homogeneity" is defined as the percentage of pores that are larger than 0.5 times the average of pore diameter and smaller than 2 times the average pore diameter. The image analyzer also calculates the porosity which is the proportion of the entire bread cross section consisting of pores.

[0066] By using image analysis it has been found that baked products made from a dough as defined above including a fat-free dough, which has been supplemented by addition of the polypeptide in accordance with the present invention attain/obtain an increased pore homogeneity and reduced pore size whereas the porosity is unchanged.

[0067] Thus, by using the polypeptide according to the invention in a fat-free dough there is provided baked products with a fortified crumb structure. It has also been found that the increased pore homogeneity and the decrease in pore size is not accompanied by a reduction of other bread characteristics such as bread volume and anti-staling properties.

[0068] As already mentioned, a most interesting characteristic of the polypeptide for use in accordance with the

## EP 1 193 314 B1

present invention is its ability to modify by hydrolysis the glycolipids, monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG), to the more polar components monogalactosyl monoglyceride (MGMG) and digalactosyl monoglyceride (DGMG) which are more surface active components than MGDG and DGDG.

[0069] Without being bound by theory, it is assumed that the improved pore homogeneity of the bread crumb which is obtained by using the polypeptide of the invention in a dough is caused by the formation of the more surface active glycerides MGMG and DGMG which in combination with the released fatty acids in ionised form will contribute to the formation of mesomorphic phases of lamellar structure.

[0070] In accordance with the invention the amount of polypeptide added to the dough corresponds to a lipase activity in the range of 100-30,000 lipase units (LUS) per kg flour, such as in the range of 500-10,000 lipase units (LUS) per kg flour including in the range of 1,000-8,000 lipase units (LUS) per kg flour.

[0071] In an interesting embodiment, the use of the invention involves combined use of the polypeptide for use in accordance with the invention and an emulsifier such as mono- and diglycerides, sorbitan esters, polysorbates, sucrose esters, citric acid esters of mono- and diglycerides, polyglycerol esters of fatty acids, propylene glycol monostearate, lactic acid esters, lecithins, mono- and diglycerides of edible fatty acids and diacetyl tartaric acid ester of mono- and diglycerides of edible fatty acids.

[0072] Diacetyl tartaric acid esters of mono- and diglycerides of edible fatty acids are well-known in the food processing technology and are widely used in the baking industry as dough additives to provide dough stability and increased volume of the baked products. Typically, diacetyl tartaric acid esters of mono- and diglycerides are used in an amount of up to 1% by weight of the flour.

[0073] It has been found that by using the polypeptide in accordance with the invention in combination with diacetyl tartaric acid esters of mono- and diglycerides of fatty acids an improved pore homogeneity is still obtained and furthermore, that a much lower concentration of diacetyl tartaric acid esters of mono- and diglycerides of fatty acids is required to obtain the same bread volume.

[0074] According to the invention an expedient amount of diacetyl tartaric acid esters of mono- and diglycerides of fatty acids is in the range of 0.1 to 1.0% by weight of the flour, preferably in the range of 0.1 to 0.5% by weight of the flour and most preferred in the range of 0.1 to 0.4% by weight of the flour.

[0075] The diacetyl tartaric acid esters of mono- and diglycerides which can be used according to the invention are characterised by having a saponification value in the range of 300 to 600, preferably a saponification value in the range of 300 to 400, and an acid value in the range of 40 to 120, preferably an acid value in the range of 50 to 100.

[0076] In accordance with the above description of the use of the polypeptide in a flour dough including a fat-free dough, the present invention relates in a further aspect to a method of preparing a baked product having improved pore homogeneity and reduced pore diameter from a dough including a fat-free dough as defined above, comprising adding the polypeptide of the invention to the dough.

[0077] In one embodiment the use of the invention relates to a polypeptide having lipase activity which has the capability of increasing the level of ethylesters of fatty acids in a flour dough by at least 10% such as by at least 50% including by at least 100%.

[0078] In accordance with the use of the invention, the polypeptide having lipase activity has the capability increasing the gluten index in a flour dough. Accordingly, in one useful embodiment, the use of the invention for preparing a baked product comprises adding to the dough the polypeptide in an amount that results in an increase of the gluten index, as determined by means of a Glutomatic 2200 apparatus, in the dough of at least 5%, relative to a dough without addition of the polypeptide.

[0079] Preferably, the gluten index is increased by at least 10%, such as at least 15% or more preferably, by at least 20%.

[0080] In another useful embodiment, the use according to the invention is one wherein at least one further enzyme is added to the dough. Examples of such further enzymes include hemicellulases, proteases, amylases, oxidoreductases such as e.g. hexose oxidase and cellulases.

[0081] The polypeptide may conveniently be added to the dough or to any of the dough ingredients or to any mixture of the dough ingredients in the form of a dry composition or as a liquid preparation comprising the polypeptide of the present invention.

[0082] As stated above the amount of polypeptide activity is in the range of 100-30,000 lipase units (LUS) per kg flour, including in the range of 500-10,000 lipase units (LUS) per kg flour such as in the range of 1,000-8,000 lipase units (LUS) per kg flour.

[0083] In a useful embodiment of the use according to the invention the polypeptide is added to the dough in admixture with a diacetyl tartaric acid ester of mono- and diglycerides of edible fatty acids.

[0084] In another useful embodiment of the use according to the invention the baked product is toast bread.

## EP 1 193 314 B1

## BRIEF DESCRIPTION OF THE FIGURES

[0085] The present invention is further illustrated by reference to the accompanying figures in which

Fig. 1 shows the restriction map of the genomic clone of the *lipA* gene.

Fig. 2 shows the structure of the *lipA* gene encoding lipase 3.

Fig. 3 shows a chromatogram of HIC fractionated culture supernatant of an *Aspergillus tubigensis* transformant with 62-fold increase of lipase 3, and

Fig. 4 shows a chromatogram of HIC fractionated culture supernatant of the untransformed *Aspergillus tubigensis* strain.

## EXAMPLES

Analytical methods for determining lipase activity and protein(I) Determination of lipase activity1. Plate assay on tributyrin-containing medium

[0086] The assay is modified from Kouker and Jaeger (Appl. Environ. Microbiol., 1987, 53:211-213).

[0087] A typical protocol for this assay is as follows: 100 ml 2% agar in 50 mM sodium phosphate buffer (pH 6.3) is heated to boiling, and after cooling to about 70°C under stirring, 5 ml 0.2% Rhodamine B is added under stirring plus 40 ml of tributyrin. The stirring is continued for 2 minutes. The mixture is then sonicated for 1 minute. After an additional 2 minutes of stirring, 20 ml of the agar mixture is poured into individual petri dishes. In the absence of lipase activity, the agar plates containing tributyrin and Rhodamine B will appear opaque and are pink coloured.

[0088] To quantify lipase activity, holes having a diameter of 3 mm are punched in the above agar and filled with 10 µl of lipase preparation. The plates are incubated for varying times at 37°C. When lipase activity is present in the applied preparation to be tested, a sharp pink/reddish zone is formed around the holes. When the plates are irradiated with UV light at 350 nm, the lipase activity is observed as halos of orange coloured fluorescence.

2. Modified Food Chemical Codex assay for lipase activity

[0089] Lipase activity based on hydrolysis of tributyrin is measured according to Food Chemical Codex, Forth Edition, National Academy Press, 1996, p. 803. With the modification that the pH is 5.5 instead of 7. One LUT (lipase unit tributyrin) is defined as the amount of enzyme which can release 2 µmol butyric acid per min. under the above assay conditions.

3. p-nitrophenyl acetate assay

[0090] Lipase activity can also be determined colorimetrically using p-nitrophenyl acetate as a substrate e.g. using the following protocol: In a microtiter plate 10 µl of sample or blank is added followed by the addition of 250 µl substrate (0.5 mg p-nitrophenyl acetate per ml 50 mM phosphate buffer, pH 6.0). The microtiter plate is incubated for 5 minutes at 30°C and the absorbance at 405 nm is read using a microplate reader. 1 unit is defined as 1 µmol p-nitrophenol released per 5 minutes.

4. p-nitrophenyl hexanoate assay

[0091] Lipase activity can be determined by using p-nitrophenyl hexanoate as a substrate. This assay is carried out by adding 10 µl of sample preparation or blank to a microtiter plate followed by the addition of 250 µl substrate (0.5 mg p-nitrophenyl hexanoate per ml of 20 mM phosphate buffer, pH 6.). At this concentration of substrate the reaction mixture appears as a milky solution. The microtiter plate is incubated for 5 minutes at 30°C and the absorbance at 405 nm is read in a microplate reader.



## EP 1 193 314 B1

5. Titrimetric assay of lipase activity

[0092] Alternatively, lipase activity is determined according to Food Chemical Codex (3rd Ed., 1981, pp 492-493) modified to sunflower oil and pH 5.5 instead of olive oil and pH 6.5. The lipase activity is measured as LUS (lipase units sunflower) where 1 LUS is defined as the quantity of enzyme which can release 1  $\mu$ mol of fatty acids per minute from sunflower oil under the above assay conditions.

6. Protein measurement

[0093] During the course of purification of lipase as described in the following, the protein eluted from the columns was measured by determining absorbance at 280 nm. The protein in the pooled samples was determined in microtiter plates by a sensitive Bradford method according to Bio-Rad (Bio-Rad Bulletin 1177 EG, 1984). Bovine serum albumin was used as a standard.

## EXAMPLE 1

Production, purification and characterization of lipase 31.1. Production

[0094] A mutant strain of *Aspergillus tubigensis* was selected and used for the production of wild type lipase. This lipase is referred to herein as lipase 3. The strain was subjected to a fermentation in a 750 l fermenter containing 410.0 kg of tap water, 10.8 kg soy flour, 11.1 kg ammonium monohydrogenphosphate, 4.0 kg phosphoric acid (75%), 2.7 kg magnesium sulfate, 10.8 kg sunflower oil and 1.7 kg antifoam 1510. The substrate was heat treated at 121°C for 45 minutes. The culture media was inoculated directly with  $7.5 \times 10^9$  spores of the mutant strain. The strain was cultivated for three days at 38°C, pH controlled at 6.5, aeration at 290 l/min and stirring at 180 rpm the first two days and at 360 rpm the last day. The fermentate was separated using a drum filter and the culture filtrate was concentrated 3.8 times by ultrafiltration. The concentrated filtrate was preserved with potassium sorbate (0.1%) and sodium benzoate (0.2%) and used as a starting material for purification of lipase.

1.2. Purification of lipase

[0095] A 60 ml sample of ferment (cf. 1.1) containing 557 LUS/ml, pH 5.5 was first filtered through a GF/B filter and subsequently through a 0.45  $\mu$ m filter. The filtered sample was desalted using a Superdex G25 SP column (430 ml, 22 x 5 cm) equilibrated in 20 mM triethanolamine, pH 7.3. The flow rate was 5 ml/min. The total volume after desalting was 150 ml.

[0096] The desalted sample was applied to a Source Q30 anion exchanger column (100 ml, 5x5 cm) equilibrated in 20 mM triethanolamine, pH 7.3. The column was washed with equilibration buffer until a stable baseline was obtained. Lipase activity was eluted with a 420 ml linear gradient from 0 to 0.35 M sodium chloride in equilibration buffer, flow rate 5 ml/min. Fractions of 10 ml were collected. Sodium acetate (100  $\mu$ l of a 2M solution) was added to each fraction to adjust pH to 5.5. Fractions 26-32 (70 ml) were pooled.

[0097] To the pool from the anion exchange step was added ammonium sulfate to 1 M and the sample was applied to a Source Phenyl HIC column (20 ml, 10x2 cm) equilibrated in 20 mM sodium acetate (pH 5.5), 1 M ammonium sulfate. The column was washed with the equilibration buffer. Lipase was eluted with a 320 ml linear gradient from 1 M to 0 M ammonium sulfate in 20 mM sodium acetate (pH 5.5), flow 1.5 ml/min. Fractions of 7.5 ml were collected.

[0098] Fractions 33-41 were analyzed by SDS-PAGE using a NOVEX system with precast gels. Both electrophoresis and silver staining of the gels were done according to the manufacturer (Novex, San Diego, USA). (The same system was used for native electrophoresis and isoelectric focusing). It was found that fraction 40 and 41 contained lipase as the only protein.

1.3. Characterization of the purified lipase

## (i) Determination of molecular weight

[0099] The apparent molecular weight of the native lipase was 37.7 kDa as measured by the above SDS-PAGE procedure. The purified lipase eluted at a molecular weight of 32.2 kDa from a Superose 12 gel filtration column (50 mM sodium phosphate, 0.2 M sodium chloride, pH 6.85, flow 0.65 ml/min) and is therefore a monomer.

[0100] The molecular weight of the lipase was also determined by matrix-assisted laser desorption ionisation (MALDI)

## EP 1 193 314 B1

by means of a time-of-flight (TOF) mass spectrometer (Voyager Bio-Spectrometry Workstation, Perspective Biosystems). Samples were prepared by mixing 0.7  $\mu$ l of desalted lipase solution and 0.7  $\mu$ l of a matrix solution containing sinapic acid (3,5-dimethoxy-4-hydroxy cinnamic acid) in 70% acetonitrile (0.1% TFA, 10 mg/ml). 0.7  $\mu$ l of the sample mixture was placed on top of a stainless steel probe tip and allowed to air-dry prior to introduction into the mass spectrometer. Spectra were obtained from at least 100 laser shots and averaged to obtain a good signal to noise ratio. The molecular mass for the lipase was found to be 30,384 Da and 30,310 Da by two independent analyses.

[0101] Digestion of the lipase with endo- $\beta$ -N-acetyl-glucosamidase H (10  $\mu$ l) from *Streptomyces* (Sigma) was carried out by adding 200  $\mu$ l lipase and incubating at 37°C for 2 hours. The digestion mixture was desalted using a VSWP filter and analyzed directly by MALDI mass spectrometry. A major component of deglycosylated lipase gave a mass of 29,339 Da and 29,333 Da by two independent analyses. A minor component with a mass of 29,508 Da was also observed. These values corresponds well to the later calculated theoretical value of 28,939 Da based on the complete amino acid sequence of the mature lipase.

#### (ii) Determination of the Isoelectric point

[0102] The isoelectric point (pI) for the lipase was determined by isoelectric focusing and was found to be 4.1.

[0103] A calculation of the pI based on the amino acid sequence as determined in the following and shown as SEQ ID NO: 9 gave an estimated pI of 4.07.

#### (iii) Determination of temperature stability

[0104] Eppendorf tubes with 25  $\mu$ l of purified lipase 3 plus 50  $\mu$ l 100 mM sodium acetate buffer (pH 5.0) were incubated for 1 hour in a water bath at respectively 30, 40, 50, and 60°C. A control was treated in the same way, but left at room temperature. After 1 hour the lipase 3 activity was determined by the p-nitrophenyl acetate assay as described above.

[0105] The purified lipase had a good thermostability. It was found that the lipase maintained 60% of its activity after 1 hour at 60°C. 80% and 85% activity was maintained after 1 hour at 50°C and 40°C respectively.

#### (iv) Determination of pH stability

[0106] Purified lipase 3 (200  $\mu$ l) was added to 5 ml of 50 mM buffer solutions: (sodium phosphate, pH 8.0, 7.0 and 6.0 and sodium acetate pH 5.0, 4.0 and 3.5). The control was diluted in 5 ml of 4 mM sodium acetate pH 5.5. After four days at 20°C the residual activity was measured by the Modified Food Chemical Codex assay for lipase activity as described above. The lipase was very stable in the pH range from 4.0 to 7.0 where it maintained about 100% activity relative to the control (Table 1.1). At pH 3.5 the lipase maintained 92% activity, and at pH 8.0 95% residual activity was maintained as compared to the control.

Table 1.1.

pH stability of lipase 3		
pH	Activity (LUT/ml)	Activity (%)
Control (pH 5.5)	89.2	100
3.5	82.5	92
4.0	91.7	103
5.0	86.5	97
6.0	92.4	104
7.0	90.6	102
8.0	84.4	95

#### EXAMPLE 2

##### Amino acid sequencing of lipase 3

[0107] Purified lipase enzyme was freeze-dried and 100  $\mu$ g of the freeze-dried material was dissolved in 50  $\mu$ l of a mixture of 8 M urea and 0.4 M ammonium hydrogencarbonate, pH 8.4. The dissolved protein was denatured and reduced for 15 minutes at 50°C following overlay with nitrogen and addition of 5  $\mu$ l 45 mM dithiothreitol. After cooling

## EP 1 193 314 B1

to room temperature, 5 µl of 100 mM iodoacetamide was added for the cysteine residues to be derivatized for 15 minutes at room temperature in the dark under nitrogen.

[0108] 135 µl of water and 5 µg of endoproteinase Lys-C in 5 µl of water was added to the above reaction mixture and the digestion was carried out at 37°C under nitrogen for 24 hours. The resulting peptides were separated by reverse phase HPLC on a VYDAC C18 column (0.46 x 15 cm; 10 µm; The Separation Group, California, USA) using solvent A: 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides were rechromatographed on a Develosil C18 column (0.46 x 10 cm, Novo Nordisk, Bagsvaerd, Denmark) using the same solvent system, prior to N-terminal sequencing. Sequencing was done using an Applied Biosystems 476A sequencer using pulsed-liquid fast cycles according to the manufacturer's instructions (Applied Biosystems, California, USA).

[0109] For direct N-terminal sequencing, the purified protein was passed through a Brownlee C2 Aquapore column (0.46 x 3 cm, 7 µm, Applied Biosystems, California, USA) using the same solvent system as above. N-terminal sequencing was then performed as described above. As the protein was not derivatized prior to sequencing, cysteine residues could not be determined.

[0110] The following peptide sequences were found:

**N-terminal:**                      **Ser-Val-Ser-Thr-Ser-Thr-Leu-Asp-Glu-**  
**Leu-Gln-Leu-Phe-Ala-Gln-Trp-Ser-Ala-**  
**Ala-Ala-Tyr-X-Ser-Asn-Asn**  
**(SEQ ID NO:1)**

**Internal peptide 1:**            **Val-His-Thr-Gly-Phe-Trp-Lys**  
**(SEQ ID NO:2)**

**Internal peptide 2:**            **Ala-Trp-Glu-Ser-Ala-Ala-Asp-Glu-Leu-**  
**Thr-Ser-Lys-Ile-Lys**  
**(SEQ ID NO:3)**

[0111] No further peptides could be purified from the HPLC fractionation assumingly because they were very hydrophobic and therefore tightly bound to the reverse phase column.

[0112] A search in SWISS-PROT database release 31 for amino acid sequences with homology to the above peptides was performed and only three sequences were found.

[0113] All of the above peptides showed a low homology to the above known sequences. Especially internal peptide 2 has very low homology to the three lipases, LIP-RHIDL, LIP-RHIMI and MDLA-PENCA from *Rhizopus delamar* (Haas and Berka, Gene, 1991, 109:107-113), *Rhizomucor miehei* (Boel et al., Lipids, 1988, 23:701-706) and *Penicillium camembertii* (Yamaguchi et al., Gene, 1991, 103:61-67; Isobe and Nokiara, Febs. Lett., 1993, 320:101-106) respectively. Although the homology was not very high it was possible to position the lipase 3 peptides on these sequences as it is shown in the below Table 2.1.

EP 1 193 314 B1

Table 2.1. Alignment of lipase 3 peptides with known lipase sequences

5	LIP_RHIDL	MVSPFISISQGVBLCLLVSSMMLGSSAVFVSGKSGSSRTAVSASDKRALPP	50
	LIP_RHIMI	MVLEQRANYLGFLIVFFTAFLV--EAVPIKROQSNSTVDS-----LPP	40
	MDLA_PENCA	KRLS-----FFAL-----SAVASLGXALPG	21
10			

40

45

50

55



## EP 1 193 314 B1

Peptide 2	IX	
LIP_RHIDL	VPSQLTAHPTFKVTVTGHSLGSAQALLAGEOLYQREPRLSFQHLSTFTVG	299
LIP_RHIMI	VLDQPKQYTPSYKVAVTGHSIAGATALLCALDLYQRESGLSSSHFLYTOG	269
MDLA_PENCA	LKEVVAQNPFXBLVVVGHSLGAAVATLAATDL--RQKQYPSAKLYAYA--	198
	. . . . . * . . . . . * . . . . . * . . . . . * . . . . .	
LIP_RHIDL	GPRVGNPTFAFYVESTGIPFQRTVHKRDIVPHVPPQSFOFLHPGVESWIK	349
LIP_RHIMI	QPRVGDPAFANTVYSTGIPYRTVNERDIVPHLPAAFGFLHAGEEYWT	319
MDLA_PENCA	SPRVGNARLAKYITAQGRNY-RFTHTEDFVFKLPILLAMTYVHVSEFYWT	247
	****. . . . . * . . . . . * . . . . . * . . . . . * . . . . .	
LIP_RHIDL	SGTEN-V-----QICTSEIETKDCSHSIVFFTSILD-HLSYF-PINEGSC	391
LIP_RHIMI	DNSPETV-----QVCTSDLETSDCSHSIVFFTSVLD-HLSYF-GINTOLC	362
MDLA_PENCA	SPNNATVSTSDIKVIDGDVSPDENTGTGLFLITDFAHIWYFVQVDAGXG	297
	. . . . . * . . . . . * . . . . . * . . . . . * . . . . .	
LIP_RHIDL	-----L	392
LIP_RHIMI	-----T	363
MDLA_PENCA	PGLPFXRV	305

## EXAMPLE 3

Isolation and purification of *Aspergillus tubigensis* genomic DNA

[0114] The *Aspergillus tubigensis* mutant strain was grown in PDB (Difco) for 72 hours and the mycelium was harvested. 0.5-1 g of mycelium was frozen in liquid nitrogen and ground in a mortar. Following evaporation of the nitrogen, the ground mycelium was mixed with 15 ml of an extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM  $\beta$ -mercaptoethanol) and 1 ml 20 % sodium dodecylsulfate. The mixture was vigorously mixed and incubated at 65°C for 10 min. 5 ml 3M potassium acetate, (pH 5.1 adjusted with glacial acetic acid) was added and the mixture further incubated on ice for 20 min. The cellular debris was removed by centrifugation for 20 min. at 20,000 x g and 10 ml isopropanol was added to the supernatant to precipitate (30 min at -20°C) the extracted DNA. After further centrifugation for 15 min at 20,000 x g, the DNA pellet was dissolved in 1 ml TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and precipitated again by addition of 0.1 ml 3 M NaAc, pH 4.8 and 2.5 ml ethanol. After centrifugation for 15 min at 20,000 x g the DNA pellet was washed with 1 ml 70 % ethanol and dried under vacuum. Finally, the DNA was dissolved in 200  $\mu$ l TE and stored at -20°C.

## EXAMPLE 4

The generation of a fragment of the putative gene coding for lipases 3 using PCR

[0115] To obtain a fragment of the putative gene (in the following referred to as the *lipA* gene) as a tag to isolate the complete gene, a PCR amplification procedure based on the information in the isolated peptide sequences was carried out.

[0116] Degenerated primers for PCR amplification of a fragment of the lipase gene were designed based on the amino acid sequences of the isolated peptides. The following three PCR primers were synthesised:

C035: TTC CAR AAN CCN GTR TGN AC (SEQ ID NO:4)

20 mer 256 mixture, based on peptide 1 sequence VHTGFWK (Reversed).

EP 1 193 314 B1

**C036: CAR YTN TTY GCN CAR TGG****(SEQ ID NO:5)**

5 18 mer 256 mixture, based on the N-terminal sequence QLFAQW.

**C037: GCV GCH SWY TCC CAV GC****(SEQ ID NO:6)**

10

17 mer 216 mixture, based on internal peptide 2 sequence AWESAA (reversed).

[0117] The oligonucleotides were synthesised on a Applied Biosystems model 392 DNA/RNA Synthesizer. To reduce the degree of degeneracy the rare Ala codon GCA and the Ser codon TCA have been excluded in design of primer C037.

15 [0118] With these primers the desired fragments were amplified by PCR. Using these primers it was expected that a fragment of about 300 bp should be amplified provided there are no introns in the fragment.

[0119] The following PCR reactions were set up in 0.5 ml PCR tubes to amplify a putative *lipA* fragment:

20 1. 0.5 µg total genomic DNA,  
100 pmol primer C036,  
100 pmol primer C037,  
10 µl PCR Buffer II (Perkin Elmer),  
6 µl 25 mM MgCl<sub>2</sub>,  
2 µl dNTP mix (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP),  
2 units Amplitaq polymerase (Perkin Elmer), and  
25 water to a total volume of 100 µl.

30 2. 0.5 µg total genomic DNA,  
100 pmol primer C035,  
100 pmol primer C036,  
10 µl PCR Buffer II (Perkin Elmer),  
6 µl 25 mM MgCl<sub>2</sub>,  
2 µl dNTP mix (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP),  
2 units Amplitaq polymerase (Perkin Elmer), and  
water to a total volume of 100 µl.

35

[0120] The reactions were performed using the following program:

40 94°C 2 min  
94°C 1 min )  
40°C 1 min )  
72°C 1 min ) These three steps were repeated for 30  
72°C 5 min cycles  
5°C SOAK

45 [0121] The PCR amplifications were performed in a MJ Research Inc. PTC-100 Thermocycler.

[0122] In reaction 1, three distinct bands of about 300, 360 and 400 bp, respectively could be detected. These bands were isolated and cloned using the pT7-Blue-T-vector kit (Novagene). The sizes of these fragment is in agreement with the expected size provided that the fragment contains 0, 1 or 2 introns, respectively.

50 [0123] The three fragments were sequenced using a "Thermo Sequenase fluorescent labelled primer cycle sequencing Kit" (Amersham) and analyzed on a ALF sequencer (Pharmacia) according to the instructions of the manufacturer. The fragment of about 360 bp contained a sequence that was identified as a lipase and, as it contained the part of the N-terminal distal to the sequence used for primer design, it was concluded that the desired *lipA* gene fragment was obtained.

55 [0124] The sequence of the about 360 bp PCR fragment (SEQ ID NO:7) is shown in the following Table 4.1. The peptide sequence used for primer design is underlined. The remaining part of the N-terminal sequence is doubly underlined.

EP 1 193 314 B1

Table 4.1. PCR-generated putative lipA sequence

5	10	20	30	40	50	60
	taccogggggtccgattCAGTCTGATCGCCCAATGGTCTGCCCCAGCTTATTGCTCGAATA					
10	Q L F A Q W S A A A Y C E N					
	70	80	90	100	110	120
	ATATCGACTCGAAGAVTCCAACTTGACATGCACGCCCAACCTCTGCATCACTCGAGG					
15	N I D S K X S N L T C T A N A C P S V E					
20	130	140	150	160	170	180
	AGGCCAGTACCACGATGCTGCTGGAGTTGACCTGTATGTCACTCAGATCGCAGACATAG					
25	E A S T T M L L E F D L Y V T Q I A D I					
	190	200	210	220	230	240
	AGCACAGCTAATTGAACAGGACGAACGACTTTTGAGGCCACAGCCGGTTTCCTGCGCCGCG					
30	R H S - L N R T N D F W R H S R F P G R					
35	250	260	270	280	290	300
	GACAACCCCAACAAGCGGCTCGTGGTGGCTTCGGGGGAAGCAGCAGATTGAGAACTGG					
40	G Q H Q Q A A R G R L P G K Q H D - E L					
	310	320	330			
	ATTGCTAATCTTGACTTCATCCTGGAGATAACG					
45	D C - X - L H P X R -					

[0125] The finding of this sequence permitted full identification of the PCR fragment as part of the *lipA* gene. The stop codon found in the reading frame can be caused either by a PCR or a reading error or there can be an intron encoded in the fragment as a consensus intron start and ending signal (shown in bold). If the putative intron is removed a shift in reading frame will occur. However, an alignment of the deduced amino acid sequence and the fungal lipases shown in Table 2.1 suggested that the fragment was part of the desired gene.

## EP 1 193 314 B1

## EXAMPLE 5

Cloning and characterisation of the *lipA* gene5 (i) Construction of an *Aspergillus tubigensis* genomic library

[0126] *Aspergillus tubigensis* genomic DNA was digested partially with Tsp5091 (New England Biolabs Inc.). 10 µg DNA was digested in 100 µl reaction mixture containing 2 units Tsp5091. After 5, 10, 15 and 20 minutes 25 µl was removed from the reaction mixture and the digestion was stopped by addition of 1 µl 0.5 M EDTA, pH 8.0. After all four reactions had been stopped, the samples were run on a 1% agarose gel in TAE buffer (10 x TAE stock containing per litre: 48.4 g Trizma base, 11.5 ml glacial acetic acid, 20 ml 0.5 M EDTA pH 8.0). *HindIII*-digested phage Lambda DNA was used as molecular weight marker (DNA molecular weight marker II, Boehringer, Mannheim). Fragments of a size between about 5 and 10 kb were cut out of the gel and the DNA fragments were purified using Gene Clean II Kit (Bio-101 Inc.). The purified fragments were pooled and 100 ng of the pooled fragments were ligated into 1 µg *EcoRI*-digested and dephosphorylated ZAP II vector (Stratagene) in a total volume of 5 µl. 2 µl of this volume was packed with Gigapack II packing extract (Stratagene) which gave a primary library of 650,000 pfu.

[0127] *E. coli* strain XL1-Blue-MRF (Stratagene) was infected with 5x 50,000 pfu of the primary library. The infected bacteria were mixed with top agarose (as NZY plates but with 6 g agarose per litre instead of the agar) and plated on 5 NZY plates (13 cm). After incubation at 37°C for 7 hours, 10 ml SM buffer (per litre: 5.8 g NaCl, 2.0 g MgCl<sub>2</sub>·7H<sub>2</sub>O, 50 ml 1 M Tris·HCl pH 7.5, 5.0 ml of 2% (w/v) gelatine) and incubated overnight at room temperature with gently shaking. The buffer containing washed-out phages was collected and pooled. 5% chloroform was added and after vigorous mixing the mixture was incubated 1 hour at room temperature. After centrifugation for 2 minutes at 10,000 x g the upper phase containing the amplified library was collected and dimethylsulphoxide was added to 7%. Aliquots of the library was taken out in small tubes and frozen at -80°C. The frozen library contained 2.7 x 10<sup>9</sup> pfu/ml with about 6% without inserts.

(ii) Screening of the *Aspergillus tubigensis* library

[0128] 2 x 50,000 pfu were plated on large (22 x 22 cm) NZY plates containing a medium containing per litre: 5 g NaCl, 2 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 g yeast extract, 10 g casein hydrolysate, 15 g agar, pH adjusted to 7.5 with NaOH. The medium was autoclaved and cooled to about 60°C and poured into the plates. Per plate was used 240 ml of medium.

[0129] The inoculated NZY plates were incubated overnight at 37°C and plaque lifts of the plates were made. Two lifts were made for each plate on Hybond N (Amersham) filters. The DNA was fixed using UV radiation for 3 min. and the filters were hybridized as described in the following using, as the probe, the above PCR fragment of about 360 bp that was labelled with <sup>32</sup>P-dCTP using Ready-to-Go labelling kit (Pharmacia).

[0130] The filters were prehybridised for one hour at 65°C in 25 ml prehybridisation buffer containing 6.25 ml 20 x SSC (0.3 M Na<sub>3</sub>citrate, 3 M NaCl), 1.25 ml 100 x Denhard solution, 1.25 ml 10% SDS and 16.25 ml water. 150 µl 10 mg/ml denatured Salmon sperm DNA was added to the prehybridization buffer immediately before use. Following prehybridization, the prehybridisation buffer was discarded and the filters hybridised overnight at 65°C in 25 ml prehybridisation buffer with the radiolabelled PCR fragment.

[0131] Next day the filters were washed according to the following procedure: 2 x 15 min. with 2 x SSC + 0.1 % SDS, 15 min. with 1 x SSC + 0.1 % SDS and 10 min. with 0.1 x SSC + 0.1% SDS.

[0132] All washes were done at 65°C. The sheets were autoradiographed for 16 hours and positive clones were isolated. A clone was reckoned as positive only if there was a hybridisation signal on both plaque lifts of the plate in question.

[0133] Seven putative clones were isolated and four were purified by plating on small petri dishes and performing plaque lifts essentially as described above.

[0134] The purified clones were converted to plasmids using an ExAssist Kit (Stratagene).

[0135] Two sequencing primers were designed based on the about 360 bp PCR fragment. The sequencing primers were used to sequence the clones and a positive clone with the *lipA* gene encoding lipase 3 was found. The isolated positive clone was designated pLIP4.

## (iii) Characterisation of the pLIP4 clone

[0136] A restriction map of the clone was made. The above 360 bp PCR fragment contained a *SacII* site and as this site could be found in the genomic clone as well this site facilitated the construction of the map. The restriction map showing the structure of pLIP4 is shown in Fig. 1. The restriction map shows that the complete gene is present in the clone. Additionally, since promoter and terminator sequences are present, it was assumed that all the important regions



EP 1 193 314 B1

is present in the clone.

[0137] A sample of *Escherichia coli* strain DH5 $\alpha$  containing pLIP4 was deposited in accordance with the Budapest Treaty with The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 24 February 1997 under the accession number NCIMB 40863.

[0138] The gene was sequenced using cycle sequencing and conventional sequencing technology. The complete sequence (SEQ ID NO:8) is shown below in Table 5.1. The sequence has been determined for both strands for the complete coding region and about 100 bp upstream and downstream of the coding region. The sequences downstream to the coding region have only been determined on one strand and contains a few uncertainties. In Table 5.1 shown below, the intron sequences are indicated as lowercase letters and the N-terminal and the two internal peptides (peptide 1 and peptide 2) are underlined:

Table 5.1. The DNA sequence for the lipA gene and flanking sequences

1	CCNDTTAATCCCCACCGGGGTTCCCGCTCCCGGATGGAGATGGGGCCAAACTGGCAAC
61	CCCCAGTTGCGCAACGGAACACCGCCGACCCGGAACAAGGATGCGGATGAGGAGATAC
121	GGTGCCCTGATGTCATGCTGGCTTCATCTGCTATCGTGACAGTCTCTTGGGTGAATAT
181	TGTTGTCTGACTTACCCCGCTTCTTGCTTTTCCGCCCTGAGGCCCTGATGGGGAATCGC
241	GGTGGGTAAATATGATTTGGGTATAAAGCGGAGATCCGAGGTGCACTTGGATTGAGGCACT
301	GTGTGTGTGTGCTATGCAAGACCCGTTGGTTCGCAAGGTTTGGTGGCTCGATTGTTTG
361	TATACCGCAAGATGTTCTCTGGACGGTTTGGAGTGCCTTTGACAGCGCTTGCTGCGCTGG
421	GTGCTGCCGCGCCCGCACCGCTTGCTGTGCGGAGtaggtgtgcccgatgtgagatggttg
481	gatagcaactgatgaagggtgaataggtgtgtctcgacttccacgttgcgatgagttgcaattg
541	TTCCGCGCAATGGTCTGCCCGCAGCTTATTGCTCGAATAATATCGACTCGAAGCACTCCAAC
601	TTGACATGCAACGCCAAGCCCTGTTCATCAGTCCAGGAGGCCAGTACCACGATGCTGCTG
661	GAGTTCCGACCTgtatgtcaactcagatcgagacatagagcacagctantttgaacagGAC
721	GAACGACTTTGGAGGCACAGCCGTTTCTTGCGCGCGGACAAACCAACAGCGGCTCTT
781	GGTCCGCTTCCGGGGAAGCAGCAGATTGAGAACTGGATTGCTAATCTTGACTTCATCCT
841	GGAAGATAACGACGACCTCTGACACCGGCTGCAAGGTCCATACTGGTTTCTGGAAGGCATG
901	GGAGTCCGCTGCCGACCAACTGACGAGCAAGATCAAGTCTGCGATGAGCACGATTCGGG
961	CTATACCCCTATACTTCACCGGGCACAGTTTGGGCGCGCATTTGCTACGCTGGGAGCGAC
1021	AGTTCTGCGAAATGACGGATATAGCGTTGAGCTGgtgagtccttcacaaagggtgatggag
1081	cgacaatcgggaacagacagtcactagTACACCTATGGATGTCTCGAATCGGAAACTAT
1141	GCGCTGGCTGAGCATATCACCAAGTCAGGGATCTGGGCGCAACTTCOGTTTACACACTTG
	A L A B H I T S Q G S G A N F R V T H L

EP 1 193 314 B1

1201 AAGACATCGTCCCGGGTCCACCCATGGACTTTGGATTCACTCAGCCAGTCCGGAA  
N D I V F R V P P M D P G F S Q P S P E  
1261 TACTGGATCACCAGTGGCAATGGAGCCAGTGTCAAGGCGTCCGATATCGAAGTCATCGAG  
Y W I T S G N G A S V T A S D I R V I E  
1321 GGAATCAATTCAACGGCGGGAAATGCAGGCGAAGCAACGGTGAGCGTTGTGGCTCACTTG  
G I N S T A G N A G E A T V S V V A H L  
1381 TGGTACTTTTTTGGGATTTCCGAGTGCGCTGCTATAACAGACCGACTGTCTAGATTAGTGG  
W Y F P A I S E C L L  
1441 ACGGAGAGAGTGTACATAAGTANTTAGTATATAATCAGAGCAACCAGTGGGTGATGG  
1501 TGGTGAAGAAGAAGAACACATTGAGTTCCCATACGKAGCAGTAAAGCACATKKGAGGC  
1561 GCTGGTTCTCCACTTGGCAGTTGGCGGCCATCATCATCTTCTCTCTTACTTTTGGT  
1621 CCACCACAACCTCCCATCCTGCCAGCGTTCGCATCCCGGGTTGCAACAACCTATCGCCTCC  
1681 GGGGCTCCGTGGTTCTCTATATTATTCATCCGACGGCGGAGCTTTCACCCCAACCT  
1741 GCGCCCGCGCAAAATCTCCCGAGTCCGTCAACTCCCTCGAACCACCGCCCGCATCGACC  
1801 TCACGACCCCGACCGTCTGAGATYGTCCACCG

## (iv) Analysis of the sequence of the complete gene

[0139] The peptide sequences obtained could all be found in the deduced amino acid sequence (see Table 5.1) which confirms again that the sequence found is the sequence of the lipase 3 gene. The gene was designated *lipA*.

[0140] The amino acid sequence was aligned with the three fungal lipases used to align the peptide sequences. The alignment is shown in Table 5.2.

Table 5.2 Alignment of the lipase 3 sequence with known fungal lipases

LIPASE3	MPSC-----RFGVLL-----TALAA	15
MDLA_PENCA	MRLS-----FFTAL-----SAVAS	14
LIP_RHIDL	MVSFISISQGVSLCLLVSEMDLGSAAVFSKSGSSENTAVEASDNALPP	50
LIP_RHIMI	MVLKQRANYLGFLVFFTAFLV--SAVPIKQSNSTVDS-----LFP	40
	* . . .	

LIPASE3	L-----	16
MDLA_PENCA	L-----	15
LIP_RHIDL	LISRCAPPSSNKGSKSDLOASPYRMQNTWTESEGGMLTSIGKRDDMLV	100
LIP_RHIMI	LIPSRTSAPSSSPSTTDPPEARAM-----SANGPLPS----DVETK	76
	*	

## EP 1 193 314 B1

	LIPASE3	-----CAKAPAFLA-----VRSVSTSTIDELQLEAQWAAA	47
	MDLA_PENCA	-----GYALPGKLO-----SRDVSTSELDQFBEWVQYAAAS	46
5	LIP_RHIDL	GGMTLDLPDAPPISSLSSTNSASDGCKVVAATDAQIQEFTKYAGIAATA	190
	LIP_RHIMI	YGMALNATSYTDSV-----VQAMSIDGGIRAATSQHNISLTYTTLSANS	121
		.....*	
10	LIPASE3	YCSNNIDSK-DSNLTCTANACPSVVEASTIMLLEFDITNDFGGTAGFLAA	96
	MDLA_PENCA	YYEADYTAQVGOKLSCSKKNCPEVEATGATVSYDFS-DSTTDTAGYIAV	95
	LIP_RHIDL	YCRSVVP---GNKWDVCVQ---CQKIVPDGKITT---TPTSLLEDTHGYVLR	192
	LIP_RHIMI	YCRTVIP---GATWDCIH---CDA-TEDLKIIR---TWSLXYDTNARVAR	162
		*. . . . *	
15	LIPASE3	DNTNKRLLVVAFRGSSTIENWIANLDFILEDRODLCTGCKVHTGFWTAWES	146
	MDLA_PENCA	DHTNSAVVLAFRGSYSVRNWWADATYV-HTNFGLCDGLAELGFWSNWKL	144
	LIP_RHIDL	SDKQNTIYLVFRGTNSFRSAITDIVFNPBDYKPV-KSAKVHAGFLSYEQ	241
20	LIP_RHIMI	GDSEKTIYIVFRGESSIRNWIADLTFVPVSYFPV-SGTRVCKGFLDSYGS	211
		.....**	
25	LIPASE3	AADEITSKIKSAMSTYSGYTLYFTQHSIGGALATLGNTVL--RNDGY-SV	193
	MDLA_PENCA	VRDDIHKELKCSVVAQNPNYELVVVGHSLGAAVATLAATDL--RGKGYPBA	192
	LIP_RHIDL	VVRDYFPVVOEQLTAMPTTKVIVTGHSLGGAQALLAGMDLYQREPRLSPK	291
	LIP_RHIMI	VQNELVATVLDQFQYPSYKVAVTGHSLGGATLICALDLYQREGLSSS	261
		.. . . . *	
30	LIPASE3	ELVYTY--GCPRIENYALASHITSQSGANFVTHLSDIVPRVPPMDPGVS	241
	MDLA_PENCA	KLYAY--ASPRVGNALAKYITAQGN--NFRFTHHDFVFKLPLLSMGYV	238
	LIP_RHIDL	NLSIPTVGGPRVGNPTFAYYVESTGIPFQ-KTVHKRDIVPHVFPQSFGFL	340
	LIP_RHIMI	NLFLYTQGGPRVGDPAFANYVVGSTGIPYR-KTVNRDIVPHLPAAFGFL	310
		*. . . . *	
35	LIPASE3	QPSPEYWTSCNQASVTASDIEVIEGIERSTAGRAGEATVSVV---AHLNY	288
	MDLA_PENCA	HVSPEYWTSPKATVSTSDIKVIDGVSPDONTGTGLPLLTDFEAKIYV	288
	LIP_RHIDL	HPGVBSWIKSGTSH-VQICTSEIE-----TKDCSNISVPPETSLDHLGY	383
40	LIP_RHIMI	HAGEFYWTNSPFTVQVCTEDLE-----TSDCSNISVPPETSVLDHLGY	354
		. . . *	
45	LIPASE3	FFAISECL-----L	297
	MDLA_PENCA	FVQVDAGRGPGLEFFRV	305
	LIP_RHIDL	F-DINEGSC-----L	392
	LIP_RHIMI	F-GINTGLC-----T	363
		* . . .	

[0141] The above alignment shows that lipase 3 is homologous to the known lipase sequences but that the homology is not very high. Deletions or insertions in the lipase 3 sequence was not observed when comparing the sequence with these three lipases. This strengthens the probability that the putative introns have been identified correctly.

[0142] A search in SWISS-PROT release 31 database was performed and it did not lead to further sequences with higher homology than that to the above known lipases (Table 5.3).

[0143] The sequence with highest homology is a mono- diacyl lipase from *Penicillium camembertii* where the identity is found to 42 %. However the C- terminal of lipase 3 resembles the 2 lipases from Zygomycetes (*Rhizopus* and *Rhizomucor*) and not the *P. camembertii* enzyme.

EP 1 193 314 B1

Table 5.3. Alignment of coding sequences of the lipA gene and gene coding for mono-diacyl lipase from *Penicillium camemberti*

5	LIPASE3	-	MFSGRFGVLLTALAALGAAPAPLAVRSVSTSTLDELQLPQWSAAATCS	-50
10	MDLA_PENCA-		MRLSPFTAL-SAVASIGYALPQRLQSRDVTSELDQFEFWQYAAASYE	-49
	LIPASE3	-	KNIDSK-DSNLTCTANACPSVERASTTMLLEFDLTNDPGGTAGFLAADNT	-99
15	MDLA_PENCA-		ADYTAQVGDKLSCSKGNCPEVEATGATVSYDFS-DSTITDTAGYLAVDNT	-98
	LIPASE3	-	NKRLVVAFRGESTIERWLANLOFILEDNDDICTGCKVHTGFWKAWESAND	-149
20	MDLA_PENCA-		NSAVVLAPRGSYSVENWVADATPV-HINPGLCDGCLAEIGFWSEWKLVRD	-147
	LIPASE3	-	ELTSKIESAMSTYSQYTLXFTGHSLGALATLGATVLRNDGY-SVELYTY	-198
25	MDLA_PENCA-		DIKELKEVVAQNPNYELVVVGHSLGAAVATLAATDLRGKQYPSAKLYAY	-197
	LIPASE3	-	GCPRIQNYALAEHITSQSGANFRVTHLNDIVPRVPPMDPQFSQPSPEYW	-248
30	MDLA_PENCA-		ASPRVGRALAKYITAGN-NFRFTHTNDPVPKLPLLSQYVHVSPPEYW	-245
	LIPASE3	-	ITSGNGASVTASDIEVIEGINSTAGRAGHATVSVV--AHLWYFPAISEC	-295
35	MDLA_PENCA-		ITSPNRTVSTSDIKVIDGQVDFDGNRTGTGLPLLTDFRAHIWYFVQVDAG	-295
40	LIPASE3	-	L-----L	-297
	MDLA_PENCA-		KGPGLPFKRV	-305

Identity: 126 amino acids (42.42%)

[0144] The N-terminal of the mature lipase has been determined by N-terminal sequencing to be the serine residue No. 28 of the lipase 3 precursor (SEQ ID NO:9) as shown in Table 5.4 below. Hence the amino acids No. 1 to No. 27 is the signal sequence.



EP 1 193 314 B1

Table 5.4: Amino acid sequence of the precursor of lipase 3

	5	10	15	20	25	30
5						
	1	M F S G R F G V L L T A L A A L G A A A P A P L A V R S V S				
	21	T S T L D E L Q L F A Q W S A A A Y C S N N I D S K D S N L				
	61	T C T A N A C P S V E E A S T T M L L E F D L T N D P G G T				
10	91	A G F L A A D N T N K R L V V A F R G S S T I E N W I A N L				
	121	D F I L E D N D D L C T G C E V H T G F W K A W E S A A D E				
	151	L T S K I K S A M S T Y S G Y T L Y P T G H S L G G A L A T				
	181	L G A T V L R N D G Y S V E L Y T Y G C P R I G N Y A L A E				
	211	H I T S Q G S G A N F R V T H L N D I V P R V P P M D P G F				
15	241	S Q P S P E Y W I T S G N G A S V T A S D I E V I E G I N S				
	271	T A G N A G E A T V S V V A H L W Y P F A I S E C L L				

Number of residues : 297.

20

[0145] Residues 167-176 are recognised as a common motif for the serine lipases (PROSITE). The crystal structure for the *Rhizomucor miehei* serine lipase has been examined and the residues in the active site identified (Brady et al., Nature, 1990, 343:767-770; Derewanda et al., J. Mol. Biol., 1992, 227:818-839). The active site residues of *R. miehei* lipase have all been conserved in all the lipases and correspond to the following residues in lipase 3: serine 173, aspartic acid 228 and histidine 285.

25

[0146] Lipase 3 contains 7 cysteine residues. Four of these are conserved in the *P. camemberti* lipase where they form disulphide bonds (Isobe and Nokuwara, Gene, 1991, 103:61-67). This corresponds to disulphide bonds between residue 62-67 and 131-134. In addition, two cysteine residues are homologous to two C residues which forms an additional disulphide bond in *Rhizopus* and *Rhizomucor* lipases corresponding to residues 49-295.

30

[0147] Two putative N-glycosylation sites were found in lipase 3 in position 59 and 269. Neither of these are conserved in the other fungal lipases.

**EXAMPLE 6**

35

Transformation of *Aspergillus tubigensis* and overexpression of lipase 3 in *A. tubigensis*

[0148] The protocol for transformation was based on the teachings of Buxton et al. (Gene, 1985, 37:207-214), Daboussi et al (Curr. Genet., 1989, 15:453-456) and Punt and van den Hondel, (Meth. Enzym., 1992, 216:447-457).

40

[0149] A multicopy *lipA* strain was produced by transforming the pLIP4 plasmid into *Aspergillus tubigensis* strain 6M 179 using cotransformation with a hygromycin resistant marker plasmid.

45

[0150] A screening procedure used to visualise fungal lipase after ultrathin layer isoelectric focusing was adapted to screen *Aspergillus tubigensis* transformants grown on agar plates. Screening of lipase producers on agar plates was done using 2% olive oil as the substrate for the enzyme (lipase) as well as the inducer for the lipase promoter. In addition, the plates contained a fluorescent dye, Rhodamine B. In the presence of olive oil, the transformants will be induced to secrete lipase. The lipase secreted into the agar plate will hydrolyse the olive oil causing the formation of orange fluorescent colonies that is visible upon UV radiation (350 nm). The appearance of fluorescent colonies was generally monitored after 24 hours of growth. After several days of growth, the lipase producing strains could be identified as orange fluorescent strains that are visible by eye. Under this plate screening condition, the untransformed strain gave no background fluorescence and appeared as opaque pink colonies.

50

[0151] Sixteen transformants that showed orange fluorescent halos were cultivated for 8 days in shake flasks containing 100 ml of minimal medium supplemented with 1% olive oil, 0.5% yeast extract and 0.2% casamino acids. The amount of lipase secreted was quantified by applying 10 µl of cell-free culture supernatant into holes punched in olive oil- Rhodamine B agar plates and incubating the plates overnight at 37°C. Five transformants with higher lipase production were found.

55

[0152] The cell-free culture supernatants from the five transformants were desalted using NAP 5 columns (Pharmacia) and equilibrated in 1M ammonium sulfate (50 mM sodium acetate, pH 5.5). The desalted culture supernatants were fractionated by hydrophobic interaction chromatography (HIC) on a BioGel Phenyl-5 PW column (Biorad). Elution was done by a descending salt gradient of 1M to 0 M ammonium sulfate (20 mM sodium acetate, pH 5.5). A single

## EP 1 193 314 B1

discrete protein peak was observed after fractionation. The area of the protein peaks were calculated among the different transformants and compared with the untransformed strain. The best transformant showed a 62-fold increase in the amount of lipase after HIC fractionation.

[0153] A chromatogram of the HIC fractionated culture supernatant of this transformant is shown in Fig. 3 and a similar chromatogram for the untransformed strain is shown in Fig. 4.

[0154] The fraction containing the transformed lipase was freeze-dried. The transformed lipase was carboxymethylated and subjected to N-terminal amino acid sequencing of the first 15 amino acids and it was found that the sequence of the recombinant lipase was exactly the same as the native lipase indicating correct signal sequence cleavage.

[0155] The different lipase fractions collected after HIC were separated on a 12% Tris-Glycine SDS gel and silver staining revealed one protein band, confirming the homogeneity of the fractions. In addition, the crude extract showed a major lipase band as the only band that accumulated in the culture supernatant in very high amounts when the fungus was cultured in the olive oil-containing medium.

[0156] The recombinant lipase was analysed by matrix-assisted laser desorption ionisation (MALDI) by means of a time-of-flight (TOF) mass spectrometer as described hereinbefore. The molecular weight of the recombinant lipase was 32,237 Da.

[0157] Detection of N-linked oligosaccharides was achieved by digestion of the lipase with endo- $\beta$ -N-acetyl-glucosaminidase H from *Streptomyces* (Sigma). Digestion of recombinant lipase secreted into the growth medium altered the mobility of the band seen on SDS-PAGE which moved as a single band with a molecular mass of about 30 kDa.

[0158] Deglycosylated recombinant lipase generated by digestion with endoglycosidase and analysed directly by MALDI mass spectrometry gave a molecular weight of the polypeptide backbone of 29,325 Da.

## EXAMPLE 7

Construction of lipase 3 glycosylation mutants

[0159] Overexpression of lipase 3 in the *A. tubigensis* strain 6M 179 (Example 6) resulted in overglycosylation of the protein and subsequent reduction of enzyme activity.

[0160] In order to circumvent the problem of overglycosylation and loss of activity, several mutated *lipA* genes were constructed. A molecular model for the three dimensional structure of lipase 3 based on database comparison with known lipases and their solved crystal structures revealed the surface topology of the two putative N-glycosylation sites in lipase. The two possible sites responsible for glycosylation are the asparagine residues at N59 and N269. The 2 asparagine residues were changed by mutation either to a threonine residue (T) or to a glutamine residue (Q). Mutation to threonine (N>T) eliminates the amide group through which asparagine is glycosylated without altering the size of the side chain as well as retaining the polar oxygen. Mutation to glutamine (N>Q) results in an extra carbon at the side chain and retention of the amide group. Three single mutants, N59T; N269T; N269Q and two double mutants, N59TN269T and N59TN269Q, were constructed as it is described in the following.

[0161] Mutagenic primers designed to incorporate specific sequence mutations were phosphorylated using T4 polynucleotide kinase as described in the Bio-Rad M13 In Vitro Mutagenesis Kit Manual. Annealing to the single stranded DNA template took place in 20mM Tris, 50mM NaCl, 2mM MgCl<sub>2</sub> at a molar ratio of primer to template of 30:1, incubation at 30°C for about 1.5 hours. The synthesis of the second strand was accomplished by T7 DNA polymerase (0.5 units) in a reaction mixture containing 0.4 mM of each dNTP, 0.75 mM ATP, 17.5 mM Tris-Cl (pH 7.4), 3.75 mM MgCl<sub>2</sub>, 21.5 mM DDT and 4T DNA ligase (5 units) for ligation of the newly synthesized strand to the 5' end of the primers. The reaction was incubated on ice for 5 minutes, then at 25°C for 5 minutes and finally at 37°C for 30 minutes and was stopped by the addition of stop buffer (10 mM Tris pH 8, 10 mM EDTA) and freezing. Reactions were analyzed on a 1% agarose gel in TAE buffer before transformation into SURE *E.coli* cells. The transformants were analyzed by DNA sequencing.

[0162] The SURE *E.coli* transformants containing the mutated *lipA* genes were deposited in accordance with the Budapest Treaty with The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 24 March 1998 under the following accession numbers. The three single mutants, N59T: NCIMB 40931; N269T: NCIMB 40932; N269Q: NCIMB 40933 and the two double mutants, N59TN269T: NCIMB 40934; N59TN269Q: NCIMB 40935.

## EP 1 193 314 B1

## EXAMPLE 8

Transformation of *Aspergillus tubigensis* strain 3M *pyrA* with *lipA* mutants encoding lipase 3 glycosylation mutants and expression of the mutated genes

## (I) transformation procedure

[0163] Spores from the *A. tubigensis* strain 3M *pyrA* were cultivated overnight at 34°C in a shake flask containing minimal media supplemented with 2% glucose and 10mM uridine. The mycelia was harvested and resuspended in lysis buffer plus lysing enzyme. The protoplasts produced were mixed with plasmids encoding the different lipase glycosylation mutants by the co-transformation method.

(II) Expression of *lipA* genes coding for mutant lipases

[0164] The transformants were screened for production of mutant lipases. Pure cultures of transformants were propagated in liquid media in the following manner: spores were added at a density of 10<sup>6</sup>/ml and grown at 34°C for 5 days under shaking at 200 rpm. The medium for cultivation of transformants contained 100 ml minimal medium supplemented with 2% sunflower oil (inducer), 1.5% peptone, 0.2 casamino acids and 50 µg/ml ampicillin. Culture filtrate was collected each day and tested on olive oil-rhodamine plates for lipase activity. The oil-rhodamine plates were inspected for fluorescent halos (lipase activity) after an overnight incubation at 37°C. In addition, a chromogenic assay using 1,2-O-dilauryl-rac-glycero-3-glutaric acid-resorufin ester (Lipase chromogenic substrate, Boehringer Mannheim Biochemica, cat. No. 1179934) was used to double check the filtrates collected on days 2, 3, 4 and 5. Table 8.1 below shows lipase activity of selected transformants expressing different modified *lipA* genes coding for mutant lipase 3. Lipase activity was measured on day 5 using the above chromogenic assay.

Table 8.1.

Lipase activity of lipase 3 enzymes encoded by glycosylation mutants of the <i>lipA</i> gene		
Transformant	Lipase type	Lipase activity
160	N59T	0.478
161	N59T	1.327
S2-2	N269Q	0.840
S2-5	N269Q	0.102
S2-6	N269Q	0.916
S2-7	N269Q	0.127
S2-8	N269Q	0.100
S3-1	N269T	1.048
S3-3	N269T	0.145
S3-4	N269T	2.86
S3-5	N269T	0.068
S3-6	N269T	0.086
S3-8	N269T	0.254
S4-1	N59TN269Q	1.088
S4-2	N59TN269Q	0.948
S4-3	N59TN269Q	1.75
S4-5	N59TN269Q	0.358
S4-7	N59TN269Q	0.097
24-6M	N59TN269T	0.424
93-10M	N59TN269T	0.249

## EP 1 193 314 B1

Table 8.1. (continued)

Lipase activity of lipase 3 enzymes encoded by glycosylation mutants of the <i>lipA</i> gene		
Transformant	Lipase type	Lipase activity
S7-10M	N59TN269T	0.263
720-4 3M	N59TN269T	0.479
Strain 6M 179	overglycosylated	0.688
<i>A. tubigensis</i> 3M <i>pyr A</i>	untransformed	0.053

[0165] In addition, lipase activity in the culture filtrates of transformants S3-4, S4-3 and strain 6M 179 (expressing over-glycosylated lipase 3) showed increasing lipolytic activities (Table 8.2) during fermentation from day 2 to day 5.

Table 8.2.

Lipase activity in culture filtrates during fermentation (chromogenic assay measured at 572 nm)				
Strain	Day 2	Day 3	Day 4	Days
S3-4	0.592	0.985	1.276	2.86
S4-3	0.176	0.452	0.680	1.75
6M 179		0.443	0.672	0.688

## EXAMPLE 9

## Fermentation and purification of wild-type and modified lipase 3

## (I) Fermentation

[0166] Spores ( $10^6$ /ml) of *Aspergillus tubigensis* strains expressing different lipase 3 glycosylation mutants were used to inoculate shake flasks containing 200 ml of minimal medium supplemented with 2% sunflower oil (inducer), 1.5% peptone, 0.2% casamino acids and 50 µg/ml ampicillin. The cultures were grown with shaking at 200 rpm for 4 days at 34°C. The culture filtrate was separated from the mycelia by filtration through nylon gauze.

## (II) Purification of lipase

[0167] Samples of culture supernatant of S2-6, S3-4, S4-1 and S4-3 fermentations, respectively were all treated in the same manner. A 15 ml sample was first desalted on a PD-10 column equilibrated in 20 mM triethanolamine, pH 7.3. The desalted sample was applied to a Source Q15 (HR5/5) anion exchanger equilibrated in 20 mM triethanolamine, pH 7.3. Flow 1.5 ml/min. The column was washed with equilibration buffer until a stable baseline was obtained. Lipase activity was then eluted with a 30 ml linear gradient of from 0 to 0.53 M NaCl in equilibration buffer. Fractions of 1.5 ml were collected. The fractions were screened for activity using the plate assay on tributyrin as described above.

[0168] To the pool of lipase activity from Source Q15 was added ammonium sulfate to 1 M and the sample was applied to a Source Phenyl HIC (HR5/5) column equilibrated in 20 mM sodium acetate (pH 5.5), 1 M ammonium sulfate. The column was washed with equilibration buffer. Lipase was eluted with a linear gradient from 1 to 0 M ammonium sulfate in 20 mM sodium acetate (pH 5.5), flow 1.5 ml/min. Fractions of 0.75 ml were collected. The lipase peak fractions were totally homogeneous (one band) as examined by SDS-PAGE followed by silver staining as described above.

[0169] The four different lipase glycosylation mutants behaved the same way throughout the purification.

## (III) Determination of specific activity

[0170] The specific activity was determined for the four purified lipase mutants. Both with respect to activity on tributyrin (LUT) as described above and with respect to sun flower oil (LUS) as also described previously. Protein was determined as described as above. The results are summarized in Table 9.1



## EP 1 193 314 B1

Table 9.1.

Specific activity of mutant lipases as compared to wild-type lipase 3 and overglycosylated lipase 3.						
	Lip. 3 Wild-type	Lip. 3 over glycosylated	S2-6	S3-4	S4-1	S4-3
Protein µg/ml	7.6	10.5	27.2	24.1	25.2	26.3
LUT/ml	30	37	86	54	73.6	72.6
LUT/mg protein	3,947	3,524	3,162	2,241	2,921	2,756
LUS/ml	104	55	283	88.2	261	248
LUS/mg protein	13,684	5,238	10,404	3,660	10,357	9,415

[0171] It can be seen that the specific activity of the mutant lipases expressed in S2-6, S4-1 and S4-3 is twice the specific activity of the overglycosylated lipase 3 when measured (LUS) on sunflower oil which contains long chain fatty acids like lipids normally present in flour and dough. When measured on tributyrin (LUT) the differences in specific activity are less pronounced. This could be explained by the fact that a possible "shadowing" of the active site by overglycosylation will be less detrimental if the substrate is small like tributyrin as opposed to the long fatty acid chains in sunflower oil. The specific activity of the S3-4 lipase 3 is lower than that of overglycosylated lipase 3 (measured on sunflower oil).

[0172] When assayed in the LUS assay none of the mutants have quite as good specific activity as the wild-type lipase 3, but as shown in a following example, the enzymatic activity is higher for the glycosylation mutants than for the wild-type lipase 3 in a dough system.

#### (iv) Determination of molecular weight

[0173] The apparent molecular weight of the purified mutant lipases was determined by running the lipases on one SDS-PAGE gel. The results are shown in table 9.2.

Table 9.2.

Molecular weight of wild-type, overglycosylated and mutant lipases							
	Lip. 3 Wild-type	Lip. 3 Overglycosylated	S2-6	S3-4	S4-1	S4-3	S5
MW (kDa)	34.8	38.9	34.8	34.8	31.2	31.2	31.2

[0174] It is seen that the overglycosylated lipase gives rise to a lipase 3 molecule which has a larger molecular weight than the wild-type native lipase 3. By removing one of the two potential N-glycosylation sites as it is done in S2-6 (N269Q) and in S3-4 (N269T) a lower molecular weight was obtained, but it is still slightly higher than for the wild-type lipase 3, indicating overglycosylation at the second glycosylation site. By removing both potential N-glycosylation sites as in S4-1 and S4-3 (both N59T N269Q), a molecular weight clearly lower than that of the wild-type lipase 3 is obtained which is in agreement with a total lack of N-glycosylation in this mutant.

#### (v) Determination of the isoelectric point for lipase mutants

[0175] The isoelectric points for the 4 different lipase glycosylation mutants were determined by chromatofocusing on a Mono P (HR5/5) to be pH 4.1 for all the mutant enzymes. The column was equilibrated in 25 mM bib-tris, pH 6.10. A pH gradient was generated by running 100% of a 10% polybuffer 74 in water (pH 3.8 with HCl) plus 2.5% betaine, this eluted the lipase(s). Flow 0.70 ml/min. Fractions of 0.4 ml were collected.

#### (vi) Determination of temperature stability of mutant lipase S4-1

[0176] A sample of purified lipase S4-1 (296 LUT/ml) was diluted 1:1 with 200 mM NaAc, pH 5.0. Eppendorf tubes with 1 ml of this diluted sample were incubated for 1 hour in a waterbath at 30, 40, 50 and 60°C respectively. A control was treated the same way but left at room temperature. The remaining lipase activity was determined by the *p*-nitro-phenyl acetate assay as described previously. The results are shown below in Table 9.3.

## EP 1 193 314 B1

Table 9.3.

Temperature stability of mutant lipase (S4-1) compared to wild-type lipase 3	
Temperature (°C)	Mutant lipase S4-1 (activity)
20 (Control)	100%
30	107%
40	96%
50	89%
60	53%

## (vii) Determination of pH stability of mutant lipase S3-4

[0177] Samples of 200 µl purified mutant lipase S3-4 were added to 5 ml of 50 mM buffer (pH 3.5, 4.0, 5.0 NaAc and pH 6.0, 7.0, 8.0 phosphate buffer). The control was diluted in to 5.0 ml of 4 mM NaAc, pH 5.5. After 4 days at 20°C the samples were measured for residual activity by the Modified Food Chemical Codex assay for lipase activity as described above. The mutant lipase S3-4 was very stable in the pH range from 4.0 to 8.0 where it maintained about 100% activity relative to the control. At pH 3.5 the lipase maintained 88% activity as compared to the control. The results are shown in Table 9.4.

Table 9.4.

pH stability of mutant lipase S3-4		
pH	Activity (LUT/ml)	Activity (%)
Control (pH 5.5)	30.6	100
3.5	26.9	88
4.0	29.3	96
5.0	30.3	99
6.0	35.3	115
7.0	33.3	109
8.0	31.3	102

## EXAMPLE 10

## Baking experiments using lipase 3

## 10.1. Baking procedures and analytical methods

## (I) Baking procedure for Danish toast bread

[0178] Flour (Danish reform flour) 2000 g, dry yeast 30 g, salt 30 g and water corresponding to 400 Brabender units + 3%, was kneaded in a Hobart Mixer with hook for 2 min. at low speed and 10 min. at high speed. Dough temperature after kneading was 25°C. Resting time was 10 min. at 30°C. The dough was scaled 750 g per dough and rested again for 5 min at 33°C and 85% RH. After moulding on a Glimik moulder, the dough were proofed in tins for 50 min at 33°C and baked in a Wachtel oven for 40 min at 220°C with steam injection for 16 sec. After cooling, the bread was scaled and the volume of the bread was measured by the rape seed displacement method. The specific volume is calculated by dividing the bread volume (ml) by the weight (g) of the bread.

[0179] The crumb was evaluated subjectively using a scale from 1 to 5 where 1 = coarsely inhomogeneous and 5 = nicely homogeneous.

[0180] Three breads baked in tins with lid were stored at 20°C and used for firmness measurements and pore measurements by means of an Image Analyzer.

## EP 1 193 314 B1

## (ii) Baking procedure for Danish rolls

[0181] Flour (Danish reform) 1500 g, compressed yeast 90 g, sugar 24 g, salt 24 g and water corresponding to 400 Brabender units - 2% were kneaded in a Hobart mixer with hook for 2 min. at low speed and 9 min at high speed. After kneading, the dough temperature was 26°C. The dough was scaled 1350 g. After resting for 10 min. at 30°C, the dough was moulded on a Fortuna moulder after which the dough was proofed for 45 min. at 34°C and baked in a Bago oven for 18 min. at 220°C with steam injection for 12 sec. After cooling, the rolls were scaled and the volume of the rolls was measured by the rape seed displacement method. Specific volume is calculated as described above.

## 10 (iii) Determination of pore homogeneity

[0182] The pore homogeneity of the bread was measured by means of an image analyzer composed of a standard CCD-video camera, a video digitiser and a personal computer with WinGrain software. For every bread, the results of pore diameter in mm and pore homogeneity were calculated as an average of measurements from 10 slices of bread. The pore homogeneity was expressed in % of pores that are larger than 0.5 times the average of pore diameter and smaller than 2 times the average diameter.

## (iv) Determination of firmness

20 [0183] The firmness of bread, expressed as N/dm<sup>2</sup>, was measured by means of an Instron UTM model 4301 connected to a personal computer. The conditions for measurement of bread firmness were:

25	Load Cell	Max. 100 N
	Piston diameter	50 mm
	Cross head speed	200 mm/min
	Compression	25%
	Thickness of bread slice	11 mm

30 [0184] The result was an average of measurements on 10 bread slices for every bread.

## (v) Determination of gluten Index

35 [0185] Gluten index was measured by means of a Glutomatic 2200 from Perten Instruments (Sweden). Immediately after proofing, 15 g of dough was scaled and placed in the Glutomatic and washed with 500 ml 2% NaCl solution for 10 min. The washed dough was transferred to a Gluten Index Centrifuge 2015 and the two gluten fractions were scaled and the gluten index calculated according to the following equation:

40 
$$\text{Gluten index} = (\text{weight of gluten remaining on the sieve} \times 100) / \text{total weight of gluten}$$

## (vi) Extraction of lipids from dough

45 [0186] 30 g of fully proofed dough was immediately frozen and freeze-dried. The freeze-dried dough was milled in a coffee mill and passed through a 235 µm screen. 4 g freeze-dried dough was scaled in a 50 ml centrifuge tube with screw lid and 20 ml water saturated n-butanol (WSB) was added. The centrifuge tube was placed in a water bath at a temperature of 100°C for 10 min. after which the tubes were placed in a Rotamix and turned at 45 rpm for 20 min. at ambient temperature. The tubes were again placed in the water bath for 10 min. and turned on the Rotamix for another 30 min. at ambient temperature.

[0187] The tubes were centrifuged at 10,000 x g for 5 min. 10 ml of the supernatant was pipetted into a vial and evaporated to dryness under nitrogen cover. This sample was used for HPLC analysis.

55 [0188] A similar sample was fractionated on a Bond Elut Si (Varian 1211-3036). The non-polar fraction was eluted with 10 ml cyclohexan:isopropanol:acetic acid (55:45:1) and evaporated to dryness. This sample was used for GLC analysis.

## EP 1 193 314 B1

## (vii) HPLC analysis

[0189] Column: LiChrospher 100 DIOL 5  $\mu$ m (Merck art. 16152) 250x4 mm with a water jacket of a temperature of 50°C.

Mobile phases:

[0190]

A: heptan:isopropanol:n-butanol:tetrahydrofuran:isooctan:water (64.5:17.5:7.5:5:1)

B: isopropanol:n-butanol:tetrahydrofuran:isooctan:water (73:7:5:5:10)

[0191] The mobile phases contained 1 mmol trifluoroacetic acid per 1 mobile phase and were adjusted to pH 6.6 with ammonia.

[0192] Pump: Waters 510 equipped with a gradient controller.

Gradient :

Flow (ml/min)	Time (min)	A (%)	B (%)
1.0	0	100	0
1.0	25	0	100
1.0	30	0	100
1.0	35	100	0
1.0	40	100	0

[0193] Detector: CUNOW DDL21 (evaporative light-scattering); temperature 100°C; voltage: 600 volt; air flow: 6.0 l/min.

[0194] Injector: Hewlett Packard 1050; Injection volume: 50  $\mu$ l.

[0195] The samples for analysis were dissolved in 5 ml chloroform:methanol (75:25), sonicated for 10 min and filtered through a 0.45  $\mu$ m filter.

## (viii) GLC analysis

[0196] Perkin Elmer 8420 Capillary Gas Chromatograph equipped with WCOT fused silica column 12.5 m x 0.25 mm coated with 0.1  $\mu$ m stationary phase of 5% phenyl-methyl-silicone (CP Sil 8 CB from Crompack).

Carrier : Helium

Injection: 1.5  $\mu$ l with split

Detector: FID 385°C

Oven program:	1	2	3	4
Oven temperature, °C	80	200	240	360
Isothermal time, min	2	0	0	10
Temperature rate, °C/min	20	10	12	—

[0197] Sample preparation: 50 mg non-polar fraction of wheat lipids was dissolved in 12 ml heptane:pyridine (2:1) containing 2 mg/ml heptadecane as internal standard. 500  $\mu$ l of the solution was transferred to a crimp vial and 100  $\mu$ l N-methyl-N-trimethylsilyl-trifluoroacetamide was added. The mixture was allowed to react for 15 min at 90°C.

[0198] Calculation: Response factors for mono-, di- and triglycerides and free fatty acids were determined from reference mixtures of these components. Based on these response factors, the glycerides and the free fatty acids were calculated in wheat lipids.

## 10.2. Baking experiments with lipase 3 in Danish toast bread

[0199] The effect of adding lipase 3 to a dough for making Danish toast bread was evaluated. The enzyme was added as a freeze-dried preparation on maltodextrin together with the other ingredients. The results of the baking tests

## EP 1 193 314 B1

are shown in Tables 10.1 to 10.4.

Table 10.1

Lipase LUS/kg flour	0	5,000	15,000	25,000
Specific volume of bread	4.43	4.43	4.22	4.37
Firmness Day 1	35	33	32	30
Firmness Day 7	90	90	85	73

Table 10.2

Lipase LUS/kg flour	0	5,000	15,000	25,000
Average diameter of the crumb pore, mm	2.96	2.33	2.47	2.65
Homogeneity of crumb pore, %	64.9	73.8	66.0	67.1
Porosity, %	85.9	84.7	85.5	85.1
Gluten index, %	42	45.5	55	65

Table 10.3

Lipase LUS/kg flour	0	5,000	15,000	25,000
Fatty acids, %	0.090	0.148	0.218	0.241
Monoglycerides, %	0.017	0.031	0.035	0.039
Diglycerides, %	0.020	0.036	0.040	0.045
Triglycerides, %	0.790	0.714	0.673	0.622

Table 10.4

Lipase LUS/kg flour	0	5,000	15,000	25,000
Monogalactosyl diglyceride, %	0.073	0.040	0.025	0.018
Digalactosyl diglyceride, %	0.244	0.220	0.182	0.127
Digalactosyl monoglyceride, %	0.008	0.022	0.044	0.054
Phosphatidyl choline, %	0.064	0.073	0.055	0.041
Lysophosphatidyl choline, %	0.164	0.182	0.171	0.165

[0200] By the addition of up to about 5,000 LUS/kg flour of the lipase no change in bread volume was observed, but at a higher dosage of lipase 3 there was a tendency to a small but not statistically significant decrease in volume (Table 10.1).

[0201] From the results in Table 10.2 it appears that lipase 3 improved the bread crumb homogeneity and that the average diameter of the crumb pores was reduced significantly.

[0202] The gluten index also clearly correlated to the addition of lipase 3 as an indication of a more firm gluten caused by the modification of the wheat lipid components causing better dough stability and a more homogeneous bread pore structure. However, these modifications appeared to be optimal at the addition of 5,000 LUS/kg flour of lipase 3 whereas a higher dosage resulted in a too strong modification of the wheat gluten.

[0203] The results of the GLC and HPLC analyses (Table 10.3) clearly demonstrated that the triglycerides in the dough were hydrolysed. But more interestingly, there was also observed a modification of the glycolipids, monogalactosyl diglyceride and digalactosyl diglyceride. These components were converted to the more polar components monogalactosyl monoglyceride and digalactosyl monoglyceride. As digalactosyl monoglyceride is a more surface active



## EP 1 193 314 B1

component than digalactosyl diglyceride it is assumed that this component contributed to the observed improved crumb cell structure and homogeneity. It also appeared that phospholipids like phosphatidyl choline were only modified to a very small extent.

5 10.3. Baking experiments with lipase 3 in Danish rolls

[0204] The effect of adding lipase 3 to a dough for making Danish rolls was evaluated. The enzyme was added as a freeze-dried preparation on maltodextrin together with the other ingredients. The results of the baking tests are shown in Tables 10.5 to 10.7.

Table 10.5

Lipase 3 LUS/kg flour	0	10,000	20,000	30,000
Specific volume of bread (45 min fermentation)	6.86	7.04	6.35	6.36
Specific volume of bread (65 min fermentation)	8.30	8.59	8.23	8.04
Subjective evaluation of crumb homogeneity	3	5	4	4

Table 10.6

Lipase 3 LUS/kg flour	0	10,000	20,000	30,000
Free fatty acids, %	0.060	0.126	0.173	0.211
Monoglycerides, %	0.028	0.050	0.054	0.063
Diglycerides, %	0.103	0.095	0.110	0.104
Triglycerides, %	0.705	0.561	0.472	0.436

Table 10.7

Lipase 3 LUS/kg flour	0	5,000	15,000	25,000
Digalactosyl diglyceride, %	0.204	0.187	0.154	0.110
Digalactosyl monoglyceride, %	0.007	0.026	0.047	0.074
Phosphatidyl choline, %	0.077	0.078	0.077	0.063
Lysophosphatidyl choline, %	0.153	0.161	0.162	0.150

[0205] It is apparent from the results shown in Table 10.5 that the addition of lipase 3 does not significantly increase the volume of the rolls. Furthermore, lipase 3 was found to improve the homogeneity of the crumb.

[0206] The GLC and HPLC analyses of the wheat lipids, as shown in Tables 10.6 and 10.7, demonstrated the modification of these lipids.

## EXAMPLE 11

Comparative baking experiments using lipase 3 and commercial lipase products

[0207] Lipase 3 was tested in Danish rolls in a comparative test with two commercially available lipase products: NOVOZYM 677 BG (no. 1885) from Novo Nordisk (Denmark) and Lipase A "Amano 6" (no. 1757) from Amano (Japan). The results are summarized in Table 11.1.

## EP 1 193 314 B1

Table 11.1

Lipase	Control	Lipase 3	No. 1885	No. 1757	No. 1757
LUS/kg flour	0	500	500	500	1,000
Specific volume of rolls	5.94	6.05	6.36	6.22	6.58

[0208] It is apparent from the results in Table 11.1 that lipase 3 has no effect on the volume of rolls whereas a significant volume effect was observed for the commercial enzyme products no. 1885 and no. 1757.

## EXAMPLE 12

Comparative experiments showing the relative hydrolysis of triglycerides compared to hydrolysis of DGDG

[0209] Lipase 3, lipase no. 1885 and lipase no. 1757 were tested in Danish rolls with the purpose of studying the relative hydrolysis of triglycerides as compared to the hydrolysis of digalactosyl diglyceride (DGDG). Fully proofed dough was freeze-dried and extracted with water saturated n-butanol. The extracted lipids were analysed by GLC and HPLC as described above. From the analytical results the degree of hydrolysis of triglycerides compared to the degree of hydrolysis of digalactosyl diglyceride (DGDG) at different levels of lipases was calculated. The levels of lipase used were for No. 1885: 0, 400 and 800 LUS/kg flour; for No. 1757: 0, 1770 and 2360 LUS/kg flour and for lipase 3: 0, 10,000 and 20,000 LUS/kg flour. The results are shown in Table 12.1.

Table 12.1

Lipase					
No. 1885		No. 1757		Lipase 3	
% triglyceride	% DGDG	% triglyceride	% DGDG	% triglyceride	% DGDG
0	0	0	0	0	0
31.8	2	14.2	1.5	20.4	8.3
43.6	3	17.1	3.6	33	24.5

[0210] From the results in Table 12.1 it is apparent that lipase 3 has a significant effect on the hydrolysis of digalactosyl diglyceride whereas the effect of the two commercial available lipases, No. 1757 and No. 1885, are negligible.

## EXAMPLE 13

Baking experiments using lipase 3 in combination with a diacetyl tartaric acid ester of mono- and diglycerides of fatty acids

[0211] Lipase 3 was tested in combination with a diacetyl tartaric acid ester of mono- and diglycerides of edible fatty acids having a saponification value of 355 and an acid value of 60 (PANODAN A2020 DATEM). The rolls were baked after four different fermentation times 45, 65, 85 and 105 minutes. Lipase 3 was added to the dough ingredients as a freeze-dried powder. Pore homogeneity was subjectively evaluated on a scale from 1 to 5 where 1 = course inhomogeneous and 5 = nice homogeneous. The results of the experiments are shown in Table 13.1.

Table 13.1

DATEM, %	0.35	0.15	0.25	0.35
Lipase 3, LUS/kg flour	0	5,000	5,000	5,000
Sp. Volume, 45 min.	4.98	4.97	5.00	5.39
Sp. Volume, 65 min.	7.00	7.44	7.33	7.88
Sp. Volume, 85 min.	7.49	7.81	8.28	8.57
Sp. Volume, 105 min.	8.06	7.99	8.87	8.91

EP 1 193 314 B1

Table 13.1 (continued)

Pore homogeneity, 105 min.	3	5	5	5
----------------------------	---	---	---	---

[0212] It appears from Table 13.1 that lipase 3 in combination with DATEM improved the pore homogeneity and it was found that there was a combined effect which means that it is possible to use DATEM at a much lower concentration in combination with lipase 3 and still obtain the same volume and a better crumb.

## EXAMPLE 14

## Baking experiment with lipase 3 in the preparation of Danish rolls with and without the addition of soy oil

[0213] The effect of purified wild-type lipase 3 was tested in the preparation of Danish rolls with and without the addition of soy oil to the dough. The baked rolls were evaluated for specific volume, crumb pore homogeneity and bread crust quality. Specific volume was measured as previously described. Pore homogeneity was subjectively evaluated on a scale from 1 to 10 where 1 = course inhomogeneous and 10 = nice homogeneous. Bread crust quality was evaluated on a scale from 1 to 10 where 1 = low quality and 10 = high quality.

[0214] Fully proofed doughs from these baking tests were frozen and freeze-dried. The freeze-dried doughs were extracted with water saturated butanol and the content of free fatty acids was determined by GLC analysis as also described in Example 10. The results from the baking tests and analysis of free fatty acids are summarized in Table 14.1.

Table 14.1.

Evaluation of roll bread quality and the content of free fatty acid in proofed dough					
Lipase 3 LUT/ kg flour	Soy oil in dough %	Specific bread volume cm <sup>3</sup> /m	Pore homogeneity	Bread crust quality	Free fatty acids ‰
0	2	7.18	2	2	1.27
1,500	2	6.94	3	4	1.65
4,500	2	6.85	7	6	2.10
13,500	2	6.38	8	5	2.84
0	0	6.60	3	2	1.34
1,500	0	6.56	3	3	1.53
4,500	0	6.31	6	6	2.04
13,500	0	5.62	7	6	2.71

[0215] From the baking experiment it appears that lipase 3 improves both the pore homogeneity and crust quality both in bread with and without added oil. By adding a high level of lipase 3 (13,500 LUT/kg flour) a reduction in bread volume was observed.

[0216] The results of the analysis for free fatty acids in dough from these baking experiments are also shown in Table 14.1. From these results it appears that lipase 3 is active in dough irrespective of whether oil is added or not. Furthermore, the level of free fatty acids in the dough was at the same level in doughs with and without oil.

## EXAMPLE 15

## Formation of ethyl ester in dough by adding lipase 3

[0217] As shown in Example 14, free fatty acids are produced when lipase 3 is added to a dough. During fermentation of a bread dough, the yeast produces carbon dioxide and ethanol, and the level of ethanol in a dough is normally in excess of 1% at the end of proofing. When lipase 3 is present in the dough, this enzyme not only catalyses the hydrolysis of triglycerides, but it was surprisingly found that ethyl ester of fatty acids is also formed.

[0218] Ethylesters of fatty acid is a well-known flavour component which is i.e. used to mask off-flavours in fat based food. Baking experiments not reported here have shown that lipase added to a dough is able to mask "old" taste of bread which occurs when bread is made from flour stored at 35°C for 3 months. This might be explained by the formation of ethyl esters in the dough.

## EP 1 193 314 B1

[0219] The amount of ethyl ester of fatty acids was determined by extracting freeze-dried dough with water saturated butanol. The isolated fat phase was analysed by GLC-MS to determine both the identity and the amount of ethyl esters of fatty acids. The results from these analyses are shown in Table 15.1

Table 15.1.

Content of ethyl ester of fatty acids in dough with added lipase	
Lipase 3, LUT/kg flour	Ethyl ester of fatty acids, ppm
0	8
1,500	9
4,500	20
13,500	93

[0220] The results clearly show an increasing level of ethyl ester of fatty acids with increasing dosage of lipase in the dough.

## EXAMPLE 16

The effect of lipase 3 in sponge cakes

[0221] The effect of lipase 3 was tested in a sponge cake made by the following recipe and procedure.

Sugar	208 g
Flour	188 g
Corn starch	60 g
Baking powder	14 g
Egg	200 g
GATODAN 504, sponge cake gel*	18 g
water	150 g
Lipase 3	see below

\*Emulsifier consisting of glycerol and propylene glycol esterified with edible fatty acids (28%), ethanol (8%), Grindsted PS 409 (25% sodium stearate in glycerol) (6%) and water (58%).

[0222] All the ingredients were mixed for 6 minutes using a Hobart N 50 mixer. The cake mix was scaled 3 x 175 g into sponge cake tins and baked for 35 minutes at 180°C.

[0223] Specific volume of the cake was measured by the rape seed displacement method and the softness of the cake was measured after storage at 20°C for 7 days using an Instron Food Tester.

[0224] The results of the baking test are summarized in Table 16.1.

Table 16.1.

Specific cake volume and softness after 7 days of storage (hPa)		
Lipase 3 (LUT/kg flour)	Specific cake volume (cm <sup>3</sup> /g)	Softness after 7 days of storage (hPa)
0	6.31	59
1,000	6.34	47
2,000	6.31	41
4,000	6.31	41
10,000	6.31	41

[0225] From the results it appears that lipase 3 improves the cake softness without changing the volume of the cake.

## EP 1 193 314 B1

## EXAMPLE 17

Baking experiment using lipase 3 and optionally hemicellulase for the preparation of rye/wheat bread.

[0226] Rye/wheat bread was made from a dough containing the following ingredients: Wheat flour, 667 g; sifted rye flour, 1333 g; "Back aroma sauer" 40 g; compressed yeast, 60 g; salt 44 g; water, 1160 g; lipase 3, see below; GRINDAMYL™ H 121 (commercial hemicellulase product of Grindsted Products, Brabrand, Denmark).

[0227] The dough was mixed using a Kemper mixer for 2 min. at low speed and for 11 min. at high speed. Dough temperature 27°C, resting time 30 min. at 32°C, scaling 800 g, proofing 20 min. at 32°C and 85% RH, baking for 30 min. at 230°C and 10 sec. steam.

[0228] Dough stickiness was evaluated using a scale from 1 to 5 where 1 = very sticky and 5 = normal not sticky. Specific bread volume was also evaluated. The results are shown in Table 16.1.

Table 17.1.

The effect of lipase 3 on stickiness and bread volume with and without addition of GRINDAMYL™ H 121			
GRINDAMYL™ H 121 ppm	Lipase 3 LUT/kg flour	Specific volume cm <sup>3</sup> /g	Dough stickiness
150	0	2,48	4
150	10,000	2,59	5
200	0	2,79	4
200	10,000	2,74	5

[0229] The addition of Lipase 3 in combination with hemicellulase to rye/wheat bread clearly improved the handling properties of the dough in that the dough was less sticky.

## EXAMPLE 18

Evaluation of the effect in model dough system of lipase 3 produced by *A. tubigensis* transformants.

[0230] Lipases produced by five *A. tubigensis* transformants: 161, S2-6, S3-4, S4-1 and 720-4 3M (see Table 8.1) were tested in a model dough system at different concentrations and the formation of free fatty acid was analyzed according to the following procedure:

[0231] A dough was prepared using the following ingredients: flour, 50 g; dry yeast, 0.375 g; NaCl, 0.75 g; water, 28 g, lipase, see below. Flour, dry yeast and salt was mixed for 1 min. in a Brabender mixing bowl (50 gram). Lipase and water was added to the dough followed by mixing for 6 min. at 62 rpm. The dough was transferred to a beaker provided with a lid and the dough was fermented 60 min. at 32°C. The dough was freeze-dried. The freeze-dried dough was grounded and sieved and extracted with water saturated butanol (WSB). WSB was evaporated under a stream of N<sub>2</sub> and the content of free fatty acids were determined by spectrophotometry according to Kwon, D.Y. and J.S. Rhee, 1996, JAOCS 83:89.

[0232] In a first experiment the purified wild-type lipase 3 was compared with the mutant enzyme produced by transformant S3-4 at different concentrations as shown in Table 18.1. The results clearly showed an increased level of fatty acid with increasing level of enzyme dosage. However it was also evident that wild-type lipase 3 levels off at about 3 % fatty acid at high lipase dosage whereas the lipase produced by transformant S3-4 continued to produce more fatty acids at increased enzyme dosage up to about 5 % fatty acid.

Table 18.1.

Release of free fatty acids in a dough model system		
LUT/kg	Lipase 3 % fatty acid	Transformant lip 3-4 % fatty acid
0	0.81	0.81
1,000	1.02	1.66
2,500	1.35	2.38



EP 1 193 314 B1

Table 18.1. (continued)

Release of free fatty acids in a dough model system		
LUT/kg	Lipase 3 ‰ fatty acid	Transformant lip 3-4 ‰ fatty acid
5,000	2.25	4.40
10,000	2.81	4.91

[0233] Lipases produced by transformants 161, S2-6, S4-1 and 720-4 3M were also tested by the same procedure with results as shown in table 18.2.

Table 18.2.

Release of free fatty acids in a dough model system				
LUT/kg flour	161 ‰ fatty acid	S2-6 ‰ fatty acid	S4-1 ‰ fatty acid	720-4 3M ‰ fatty acid
0	1.57	1.12	1.12	1.57
1,000	2.03	2.05	2.39	2.18
2,500	3.46	2.58	2.91	2.85
5,000	3.55	4.35	3.92	3.89
10,000	5.29	6.15	6.21	5.95

[0234] The results clearly demonstrated that the five different transformant lipases were more active in a dough as compared to wild-type lipase 3 when compared at the same enzyme dosage (LUT/kg).

## EXAMPLE 19

Baking experiment using lipases produced by S3-4 and S4-1 in the preparation of Danish rolls.

[0235] Baking experiments where Danish rolls were made with different dosages of lipase produced by transformant S3-4 and S4-1, respectively, was carried out. After baking, bread specific volume was determined and the crumb homogeneity was evaluated subjectively as described above. The results from the baking tests are shown in table 19.1.

Table 19.1.

The effect on bread quality of lipases produced by transformants S3-4 and S4-1, respectively			
Enzyme producing transformant	Dosage LUT/kg flour	Specific volume cm <sup>3</sup> /g	Crumb score
	0	6.52	4
S3-4	1,000	6.64	4
S3-4	2,500	6.61	5
S3-4	7,500	5.88	8
S4-1	1,000	6.08	5
S4-1	2,500	5.9	7
S4-1	5,000	5.51	9

[0236] Increased dosage of both the above lipases improved the crumb structure and produced bread with better appearance and crust structure. At high dosage of the S3-4 produced enzyme, a decrease in bread volume appeared and the same tendency was observed for the S4-1 produced lipase at a lower dosage.

[0237] It is concluded that also these tested mutant lipases improved dough and bread stability and improved the crumb structure as does the wild-type lipase 3.

EP 1 193 310 B1

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 136a)

5

10

15

20

25

30

35

40

45

50

55

A. The indications made below relate to the microorganism referred to in the description on page <u>35</u> . line <u>26</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution The National Collections of Industrial and Marine Bacteria Limited (NCIMB)	
Address of depositary institution (including postal code and country) 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY	
Date of deposit 24 February 1997	Accession Number NCIMB 40863
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is contained on an additional sheet <input type="checkbox"/>	
As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
<input checked="" type="checkbox"/> For receiving Office use only This sheet was received with the international application	<input type="checkbox"/> For International Bureau use only This sheet was received by the International Bureau on:
Authorized officer <i>John Cordis Paulsen</i> John Cordis Paulsen Head Clerk	Authorized officer

EP 1 193 314 B1

INDICATIONS RELATING TO DEPOSITED MICROORGANISMS  
(PCT Rule 12bis)

3

## Additional sheet

10

In addition to the microorganism indicated on page 80 of the description, the following microorganisms have been deposited with

15

The National Collections of Industrial and Marine  
Bacteria Limited (NCIMB)  
23 St. Marchar Drive, Aberdeen, Scotland  
United Kingdom AB2 1RY

20

on the dates and under the accession numbers as stated below:

25

Accession number	Date of deposit	Description Page No.	Description Line No.
---------------------	--------------------	-------------------------	-------------------------

30

NCIMB 40931	24 March 1998	45	1
NCIMB 40932	24 March 1998	45	1
NCIMB 40933	24 March 1998	45	1
NCIMB 40934	24 March 1998	45	2
NCIMB 40935	24 March 1998	45	3

35

40

For all of the above-identified deposited microorganisms, the following additional indications apply:

45

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms stated above only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

50

55

EP 1 193 314 B1

## SEQUENCE LISTING

[0238]

## 5 (1) GENERAL INFORMATION:

## (i) APPLICANT:

10 (A) NAME: DANISCO A/S  
(B) STREET: Langebrogade 1  
(C) CITY: Copenhagen  
(E) COUNTRY: Denmark  
(F) POSTAL CODE (ZIP): 1001 K  
(G) TELEPHONE: +45 32 66 22 00  
15 (H) TELEFAX: +45 32 66 21 67

(ii) TITLE OF INVENTION: Cloning and use of Lipase 3 gene from *Aspergillus tubigensis*

20 (iii) NUMBER OF SEQUENCES: 9

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
25 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## 30 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

40 (v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Aspergillus tubigensis*

## 45 (xi) SEQUENCE DESCRIPTION: SBQ ID NO: 1:

Ser Val Ser Thr Ser Thr Leu Asp Glu Leu Gln Leu Phe Ala Gln Trp  
1 5 10 15  
50 Ser Ala Ala Ala Tyr Xaa Ser Asn Asn  
20 25

## 55 (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

## EP 1 193 314 B1

(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

10

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Aspergillus tubigensis*

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Val His Thr Gly Phe Trp Lys  
1 5

20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

25

(A) LENGTH: 14 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

35

(A) ORGANISM: *Aspergillus tubigensis*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

40

Ala Trp Glu Ser Ala Ala Asp Glu Leu Thr Ser Lys Ile Lys  
1 5 10

45

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

50

(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

55

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:



EP 1 193 314 B1

TTCCARAANC CAGTRTGAAC

20

5 (2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

20

CARYTTTTC CACATGG

28

(2) INFORMATION FOR SEQ ID NO: 6:

25 (i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 17 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

35 (A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

40

CCVGGCHSWYT CCAVGC

17

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 317 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR fragment"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

55

## EP 1 193 314 B1

5  
10  
15  
20  
25  
30  
35  
40  
45  
50  
55

CAGTTGTTGG CGCAATGGTC TGCCGCAGCT TATTGCTGGA ATAATATCGA CTGAAAQAV 60  
TCCAACTTGA CATGCACGGC CAACGCCCTGT CCATCACTGG AGGAGGCCAG TACCACGATG 120  
CTGCTGGAGT TCGACCTGTA TGTCACTCAG ATCCCAQACA TAGAGCACAG CTAATTGAAC 180  
AGGACGAACG ACTTTTGGAG GCACAGCCGG TTCTCTGGCC GCGGACAACA CCAACAAGCG 240  
GCTCGTGGTC GCCTTCCGGG GAAGCAGCAC GATTGAGAAC TCGATTGCEA ATCTTGACTT 300  
CATCCTGGRA GATAACG 317

## (2) INFORMATION FOR SEQ ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1045 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Aspergillus tubigenis*

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
(B) LOCATION: join (1..82, 135..300, 347..683, 737..1045)

## (ix) FEATURE:

- (A) NAME/KEY: sig\_peptide  
(B) LOCATION: 1..81

## (ix) FEATURE:

- (A) NAME/KEY: mat\_peptide  
(B) LOCATION: join (62, 135..300, 347..683, 737..1042)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

## EP 1 155 144 B1

ATG TTC TCT GGA CCG TTT GGA GTG CTT TTG ACA GCG CTT GCT GCG CTG 48  
Met Phe Ser Gly Arg Phe Gly Val Leu Leu Thr Ala Leu Ala Ala Leu  
-27 -25 -20 -15

5  
GCT GCT GCC GCG CCG GCA CCG CTT GCT GTG GCG A GAGGTGTGC 92  
Gly Ala Ala Ala Pro Ala Pro Leu Ala Val Arg  
-10 -5

10  
CCGATGTGAG ATGGTTGGAT AGCACTGATG AAGGTTGAAT AG GT GTC TCG ACT 145  
Ser Val Ser Thr  
1

15  
TCC ACC TTG GAT GAG TTG CAA TTG TTC GCG CAA TGG TCT GCC GCA GCT 193  
Ser Thr Leu Asp Glu Leu Gln Leu Phe Ala Gln Trp Ser Ala Ala Ala  
5 10 15 20

20  
TAT TGC TCG AAT AAT ATC GAC TCG AAA GAC TCC AAC TTG ACA TGC ACG 241  
Tyr Cys Ser Asn Asn Ile Asp Ser Lys Asp Ser Asn Leu Thr Cys Thr  
25 30 35

25  
GCC AAC GCC TGT CCA TCA GTC GAG GAG GCC AGT ACC ACG ATG CTG CTG 289  
Ala Asn Ala Cys Pro Ser Val Glu Glu Ala Ser Thr Thr Met Leu Leu  
40 45 50

30

35

40

45

50

55

## EP 1 193 314 B1

5  
55  
340  
GAG TTC GAC CT GATGTCACCT CAGATCGCAG ACATAGAGCA CAGCTAATT  
Glu Phe Asp Leu

10  
60 65 70  
389  
GAACAG G ACG AAC GAC TTT GGA GGC ACA GCC GGT TTC CTG GCC GCG GAC  
Thr Asn Asp Phe Gly Gly Thr Ala Gly Phe Leu Ala Ala Asp

15  
75 80 85  
437  
AAC ACC AAC AAG CCG CTC GTG GTC GCC TTC CCG GGA AGC AGC ACG ATT  
Asn Thr Asn Lys Arg Leu Val Val Ala Phe Arg Gly Ser Ser Thr Ile

20  
90 95 100  
485  
GAG AAC TGG ATT GCT AAT CTT GAC TTC ATC CTG GAA GAT AAC GAC GAC  
Glu Asn Trp Ile Ala Asn Leu Asp Phe Ile Leu Glu Asp Asn Asp Asp

25  
105 110 115  
523  
CTC TGC ACC GGC TGC AAG GTC CAT ACT GGT TTC TGG AAG GCA TGG GAG  
Leu Cys Thr Gly Cys Lys Val His Thr Gly Phe Trp Lys Ala Trp Glu

30  
120 125 130  
581  
TCC GCT GCC GAC GAA CTG ACG AGC AAG ATC AAG TCT GCG ATG AGC ACG  
Ser Ala Ala Asp Glu Leu Thr Ser Lys Ile Lys Ser Ala Met Ser Thr

35  
135 140 145 150  
629  
TAT TCG GGC TAT ACC CTA TAC TTC ACC GCG CAC AGT TTG GCG GGC GCA  
Tyr Ser Gly Tyr Thr Leu Tyr Phe Thr Gly His Ser Leu Gly Gly Ala

40  
155 160 165  
677  
TTG GCT ACG CTG GGA GCG ACA GTT CTG CGA AAT GAC GGA TAT AGC GTT  
Leu Ala Thr Leu Gly Ala Thr Val Leu Arg Asn Asp Gly Tyr Ser Val

45  
170 175 180  
781  
GAG CTG GTGATTCCTT CACAAAGGTG ATGGAGCGAC AATCGGGAAC AGACAGTCAA  
Glu Leu

50  
185 190 195  
829  
TAG TAC ACC TAT GGA TGT CCT CGA ATC GGA AAC TAT GCG CTG GCT GAG  
Tyr Thr Tyr Gly Cys Pro Arg Ile Gly Asn Tyr Ala Leu Ala Glu

55  
200 205 210  
877  
CAT ATC ACC AGT CAG GGA TCT GGG GCC AAC TTC GGT GTT ACA CAC TTG  
His Ile Thr Ser Gln Gly Ser Gly Ala Asn Phe Arg Val Thr His Leu

EP 1 193 314 B1

5      ARC GAC ATC GTC CCC CGG GTG CCA CCC ATG GAC TTT GGA TTC AGT CAG      877  
Asn Asp Ile Val Pro Arg Val Pro Pro Met Asp Phe Gly Phe Ser Gln  
200                      205                      210                      215

10      CCA AOT CCG GAA TAC TGG ATC ACC AGT GGC AAT GGA GCC AGT GTC ACG      925  
Pro Ser Pro Glu Tyr Trp Ile Thr Ser Gly Asn Gly Ala Ser Val Thr  
                    220                      225                      230

15      GCG TCG GAT ATC GAA GTC ATC GAG GGA ATC AAT TCA ACC GCG GGA AAT      973  
Ala Ser Asp Ile Glu Val Ile Glu Gly Ile Asn Ser Thr Ala Gly Asn  
                    235                      240                      245

20      GCA GGC GAA GCA ACC GTG AGC GTT GTG GCT CAC TTG TGG TAC TTT TTT      1021  
Ala Gly Glu Ala Thr Val Ser Val Val Ala His Leu Trp Tyr Phe Phe  
                    250                      255                      260

25      GCG ATT TCC GAG TGC CTG CTA TAA                                      1045  
Ala Ile Ser Glu Cys Leu Leu \*  
                    265                      270

## (2) INFORMATION FOR SEQ ID NO: 9:

## (i) SEQUENCE CHARACTERISTICS:

30      (A) LENGTH: 297 amino acids  
          (B) TYPE: amino acid  
          (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

40      Met Phe Ser Gly Arg Phe Gly Val Leu Leu Thr Ala Leu Ala Ala Leu  
      -27       -25                      -20                      -15

45      Gly Ala Ala Ala Pro Ala Pro Leu Ala Val Arg Ser Val Ser Thr Ser  
      -10                      -5                      1                      5

50      Thr Leu Asp Glu Leu Gln Leu Phe Ala Gln Trp Ser Ala Ala Ala Tyr  
                    10                      15                      20

55      Cys Ser Asn Asn Ile Asp Ser Lys Asp Ser Asn Leu Thr Cys Thr Ala  
                    25                      30                      35

60      Asn Ala Cys Pro Ser Val Glu Glu Ala Ser Thr Thr Met Leu Leu Glu  
                    40                      45                      50



## EP 1 193 314 B1

Phe Asp Leu Thr Asn Asp Phe Gly Gly Thr Ala Gly Phe Leu Ala Ala  
 55 60 65  
 5 Asp Asn Thr Asn Lys Arg Leu Val Val Ala Phe Arg Gly Ser Ser Thr  
 70 75 80 85  
 Ile Glu Asn Trp Ile Ala Asn Leu Asp Phe Ile Leu Glu Asp Asn Asp  
 10 90 95 100  
 Asp Leu Cys Thr Gly Cys Lys Val His Thr Gly Phe Trp Lys Ala Trp  
 105 110 115  
 15 Glu Ser Ala Ala Asp Glu Leu Thr Ser Lys Ile Lys Ser Ala Met Ser  
 120 125 130  
 Thr Tyr Ser Gly Tyr Thr Leu Tyr Phe Thr Gly His Ser Leu Gly Gly  
 20 135 140 145  
 Ala Leu Ala Thr Leu Gly Ala Thr Val Leu Arg Asn Asp Gly Tyr Ser  
 150 155 160 165  
 25 Val Glu Leu Tyr Thr Tyr Gly Cys Pro Arg Ile Gly Asn Tyr Ala Leu  
 170 175 180  
 Ala Glu His Ile Thr Ser Gln Gly Ser Gly Ala Asn Phe Arg Val Thr  
 30 185 190 195  
 His Leu Asn Asp Ile Val Pro Arg Val Pro Pro Met Asp Phe Gly Phe  
 200 205 210  
 35 Ser Gln Pro Ser Pro Glu Tyr Trp Ile Thr Ser Gly Asn Gly Ala Ser  
 215 220 225  
 Val Thr Ala Ser Asp Ile Glu Val Ile Glu Gly Ile Asn Ser Thr Ala  
 40 230 235 240 245  
 Gly Asn Ala Gly Glu Ala Thr Val Ser Val Val Ala His Leu Trp Tyr  
 250 255 260  
 45 Phe Phe Ala Ile Ser Glu Cys Leu Leu  
 265 270

## 55 Claims

1. The use of a polypeptide having lipase activity in the preparation of a dough and/or baked product and which polypeptide is a triacylglycerol hydrolysing enzyme and wherein said polypeptide is capable of splitting off fatty

## EP 1 193 314 B1

acids having short, medium and long chain length, characterised in that said polypeptide is capable of hydrolysing galactolipids that are normally present in the flour to the corresponding galactosyl monoglycerides; wherein said polypeptide retains at least 80% activity after 4 days at 20°C and at a pH in the range of 3.5-8; and wherein said polypeptide is capable of hydrolysing at least 10% of the galactosyl diglycerides normally present in the flour dough to galactosyl monoglycerides and wherein the enzyme imparts one or more of the following characteristics on the baked product improved pore homogeneity and reduced pore diameter.

2. Use according to claim 1 wherein said pH is in the range of 5-7.
3. Use according to claim 1 or claim 2 wherein said polypeptide retains at least 60% of its activity after 1 hour at 60°C in 100mM sodium acetate buffer at pH5.0, including a polypeptide that retains at least 80% of its activity after 1 hour at 50°C under the same conditions.
4. Use according to any one of claims 1 to 3, wherein when said polypeptide is added to a bread dough in an amount of 5,000 lipase units per kg flour, the average pore diameter of the crumb of the bread made from the dough is reduced by at least 10%, relative to a bread which is made from a bread dough without addition of the lipase.
5. Use according to any one of the preceding claims, wherein when said polypeptide is added to a bread dough in an amount of 5,000 lipase units per kg flour, the pore homogeneity of the crumb of the bread made from the dough is increased by at least 5%, relative to a bread which is made from a bread dough without addition of the lipase.
6. Use according to any one of the preceding claims, wherein the dough is a fat free dough.
7. Use according to any one of the preceding claims, wherein the dough and/or baked product further comprises an emulsifier.
8. Use according to any one of the preceding claims, wherein the polypeptide is capable of hydrolysing at least 25% of the galactosyl diglycerides normally present in the flour dough to galactosyl monoglycerides.

### Patentansprüche

1. Verwendung eines Polypeptids mit Lipaseaktivität zur Herstellung eines Teiges und/oder eines gebackenen Produkts, wobei das Polypeptid ein Triacylglycerol hydrolysierendes Enzym ist und wobei das Polypeptid in der Lage ist, Fettsäuren mit kurzer, mittlerer und langer Kettenlänge zu spalten, dadurch gekennzeichnet, daß das Polypeptid in der Lage ist, Galaktolipide, die normalerweise in dem Mehl vorhanden sind, zu den entsprechenden Galaktosylmonoglyceriden zu hydrolysieren, wobei das Polypeptid wenigstens 80% Aktivität nach vier Tagen bei 20°C und bei einem pH-Wert im Bereich von 3,5 bis 8 behält und wobei das Polypeptid in der Lage ist, wenigstens 10% der Galaktosyldiglyceride, die normalerweise in dem Mehlteig vorhanden sind, zu Galaktosylmonoglyceriden zu hydrolysieren, und wobei das Enzym dem gebackenen Produkt eine oder mehrere der folgenden Eigenschaften, nämlich verbesserte Homogenität und verminderten Porendurchmesser, verleiht.
2. Verwendung nach Anspruch 1, wobei der pH-Wert im Bereich von 5 bis 7 liegt.
3. Verwendung nach Anspruch 1 oder Anspruch 2, wobei das Polypeptid wenigstens 60% von seiner Aktivität nach einer Stunde bei 60°C in 100 mM Natriumacetatpuffer bei einem pH-Wert von 5,0 behält, einschließlich einem Polypeptid, welches wenigstens 80% seiner Aktivität nach einer Stunde bei 50°C unter den gleichen Bedingungen behält.
4. Verwendung nach einem der Ansprüche 1 bis 3, wobei, wenn das Polypeptid einem Brotteig in einer Menge von 5000 Lipaseeinheiten pro kg Mehl hinzugefügt wird, der durchschnittliche Porendurchmesser der Krume des Brots, das aus dem Teig hergestellt wird, um wenigstens 10% gegenüber einem Brot, welches aus einem Brotteig ohne Zugabe der Lipase hergestellt ist, vermindert ist.
5. Verwendung nach einem der vorangegangenen Ansprüche, wobei, wenn das Polypeptid einem Brotteig in einer Menge von 5000 Lipaseeinheiten pro kg Mehl hinzugefügt wird, die Porenhomogenität der Krume des Brots, das aus dem Teig hergestellt ist, um wenigstens 5% gegenüber einem Brot, welches aus einem Brotteig ohne Zugabe der Lipase hergestellt ist, erhöht wird.

## EP 1 193 314 B1

6. Verwendung nach einem der vorangegangenen Ansprüche, wobei der Teig ein fettfreier Teig ist.
7. Verwendung nach einem der vorangegangenen Ansprüche, wobei der Teig und/oder das gebackene Produkt weiterhin ein Emulgiermittel umfaßt.
8. Verwendung nach einem der vorangegangenen Ansprüche, wobei das Polypeptid in der Lage ist, wenigstens 25% der Galaktosyldiglyceride, die normalerweise in dem Mehlteig vorhanden sind, zu Galaktosylmonoglyceriden zu hydrolysieren.

## Revendications

1. Utilisation d'un polypeptide ayant une activité de lipase dans la préparation d'une pâte et/ou d'un produit cuit au four, polypeptide qui est une enzyme hydrolysant le triacylglycérol, et dans laquelle ledit polypeptide est capable de scinder les acides gras ayant des longueurs de chaîne courtes, moyennes et longues, caractérisée en ce que en ce que ledit polypeptide est capable d'hydrolyser les galactolipides qui sont présents normalement dans la farine en les galactosyl-monoglycérides correspondants ; ledit polypeptide conservant au moins 80 % de son activité après 4 jours à 20°C à un pH compris dans la plage de 3,5 à 8 ; et ledit polypeptide étant capable d'hydrolyser au moins 10 % des galactosyl-diglycérides normalement présents dans la pâte de farine en galactosyl-monoglycérides, et l'enzyme conférant une ou plusieurs des caractéristiques suivantes au produit cuit au four : une homogénéité des pores améliorée et un diamètre des pores réduit.
2. Utilisation suivant la revendication 1, dans laquelle ledit pH est compris dans la plage de 5 à 7.
3. Utilisation suivant la revendication 1 ou la revendication 2, dans laquelle ledit polypeptide conserve au moins 60 % de son activité après 1 heure à 60°C dans du tampon à l'acétate de sodium 100 mM à pH 5,0, y compris un polypeptide qui conserve au moins 80 % de son activité après 1 heure à 50°C dans les mêmes conditions.
4. Utilisation suivant l'une quelconque des revendications 1 à 3, dans laquelle, lorsque ledit polypeptide est ajouté à une pâte à pain en une quantité de 5000 unités de lipase par kg de farine, le diamètre moyen des pores de la mie du pain préparé à partir de la pâte est réduit d'au moins 10 %, par rapport à un pain qui est préparé à partir d'une pâte à pain sans addition de la lipase.
5. Utilisation suivant l'une quelconque des revendications précédentes, dans laquelle, lorsque ledit polypeptide est ajouté à une pâte à pain en une quantité de 5000 unités de lipase par kg de farine, l'homogénéité des pores de la mie du pain préparé à partir de la pâte est augmentée d'au moins 5 %, par rapport à un pain qui est préparé à partir d'une pâte à pain sans addition de la lipase.
6. Utilisation suivant l'une quelconque des revendications précédentes, dans laquelle la pâte est une pâte sans matière grasse.
7. Utilisation suivant l'une quelconque des revendications précédentes, dans laquelle la pâte et/ou le produit cuit au four comprennent en outre un émulsionnant.
8. Utilisation suivant l'une quelconque des revendications précédentes, dans laquelle le polypeptide est capable d'hydrolyser au moins 25 % des galactosyl-diglycérides normalement présents dans la pâte de farine en galactosyl-monoglycérides.

EP 1 193 314 B1

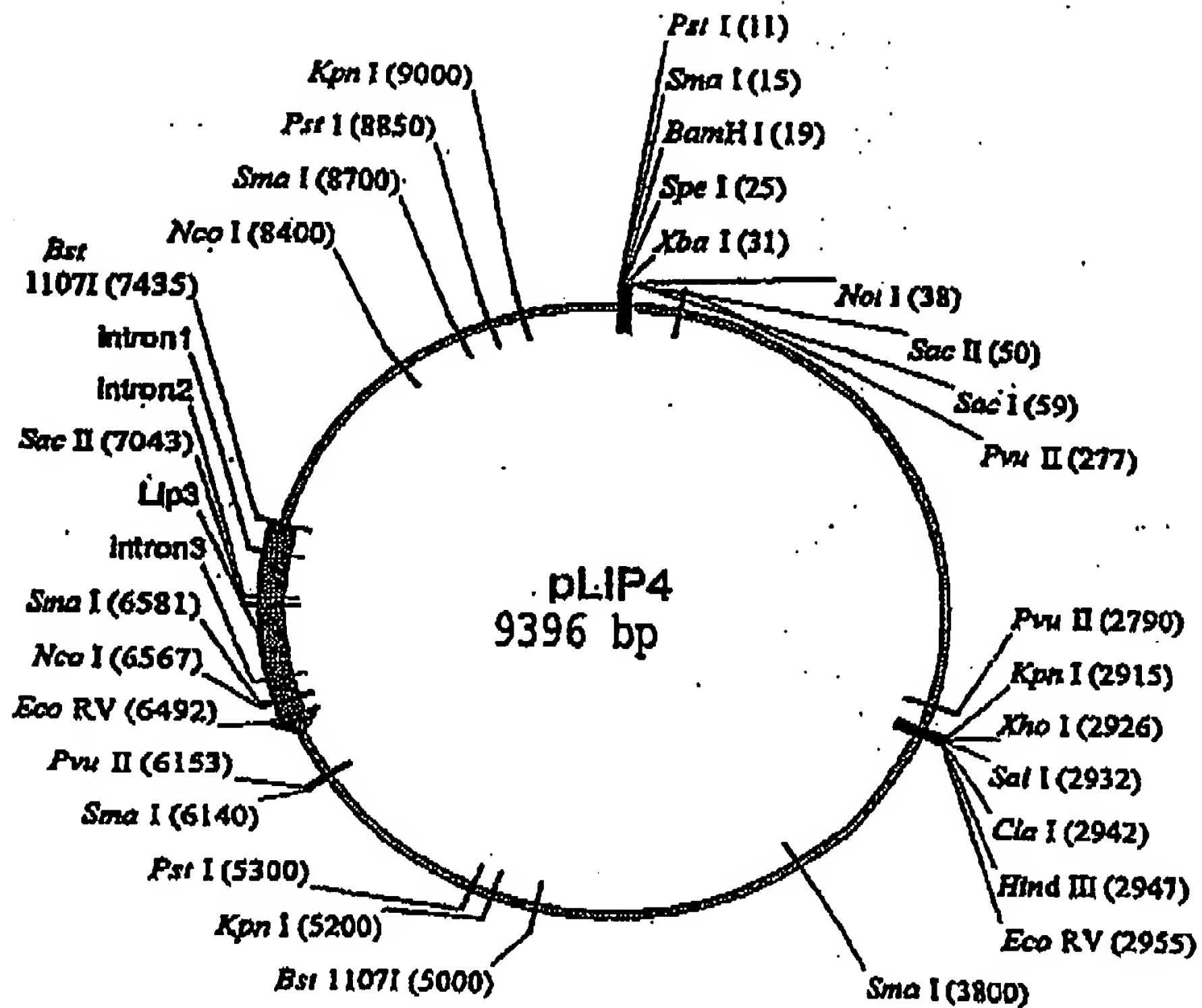


Fig. 1

EP 1 193 314 B1

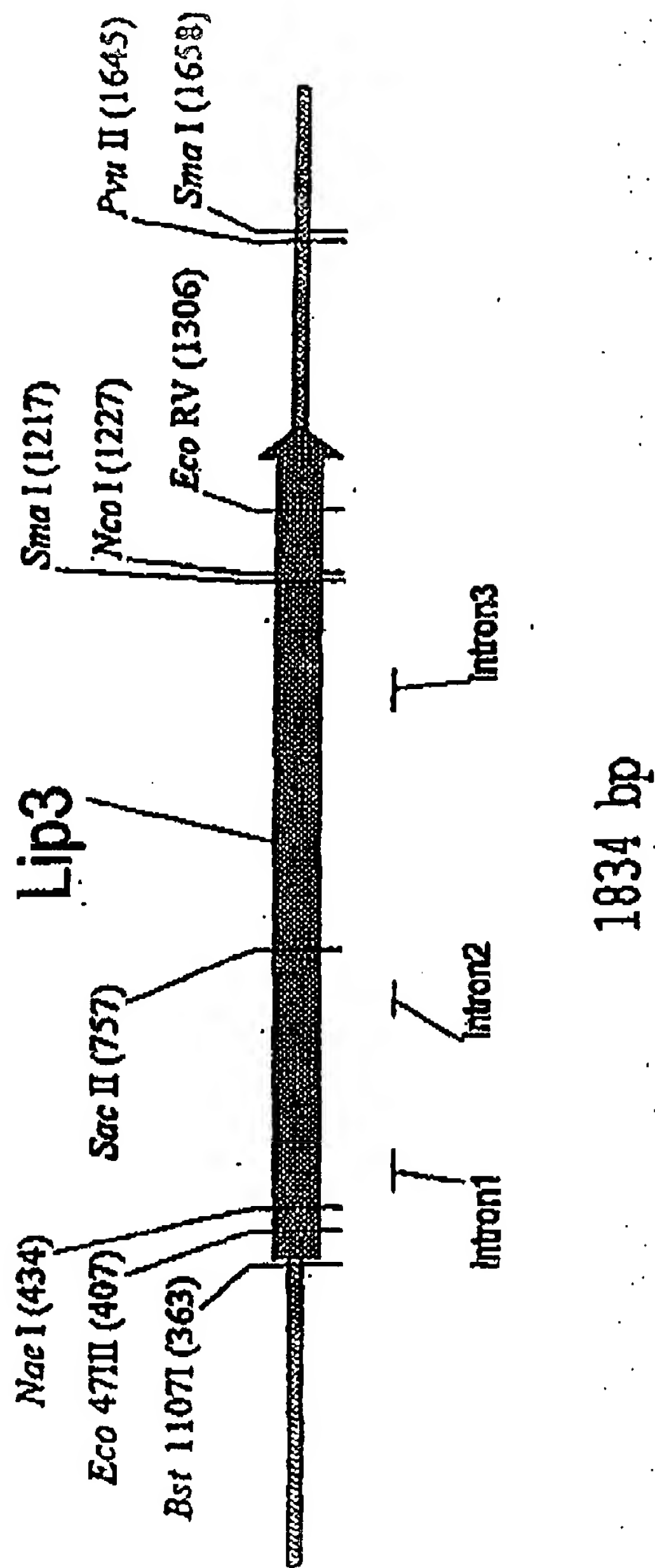


Fig. 2

EP 1 193 314 B1

# BEST TRANSFORMANT

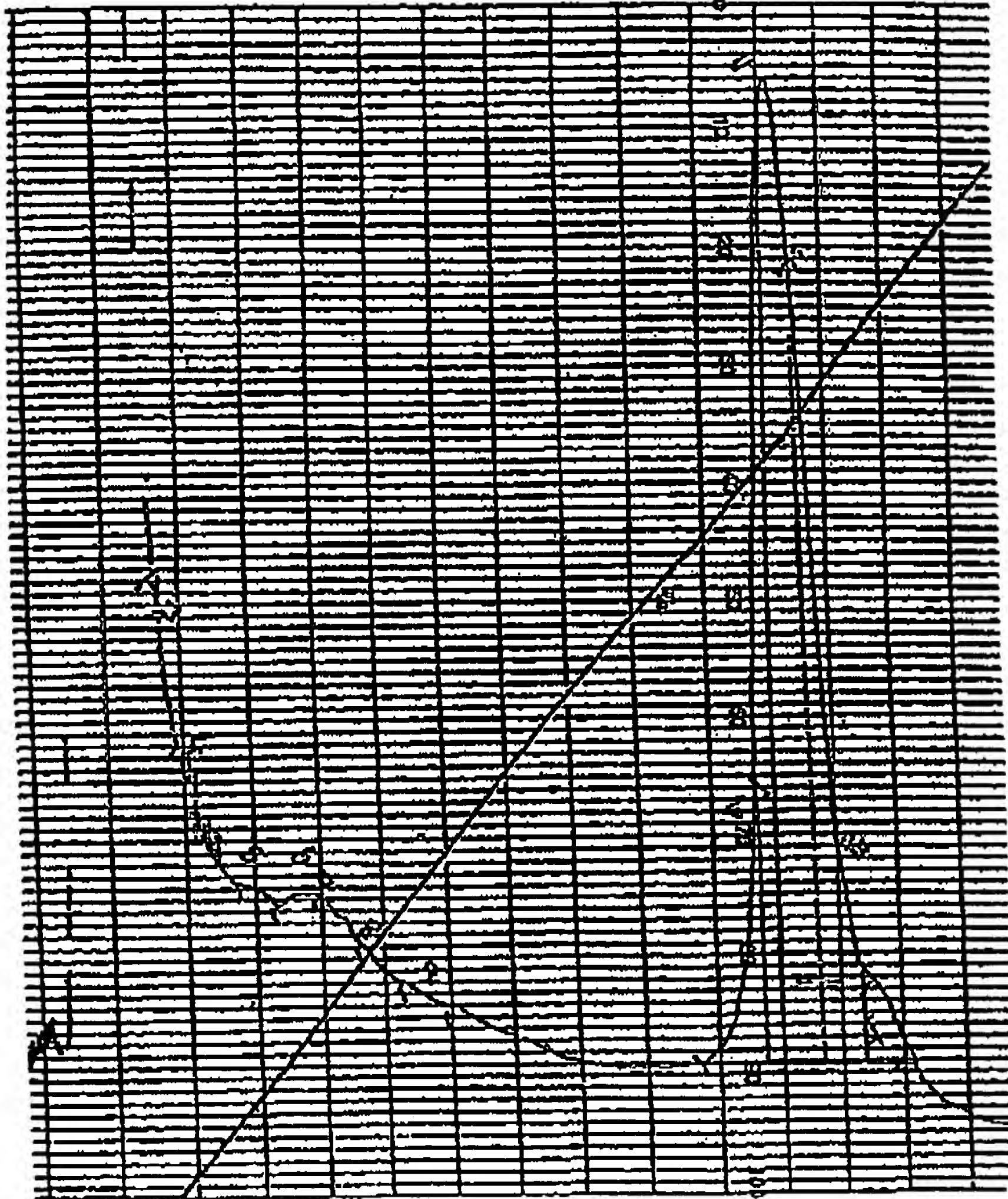


Fig. 3



EP 1 193 314 B1

## CONTROL - UNTRANSFORMED STRAIN

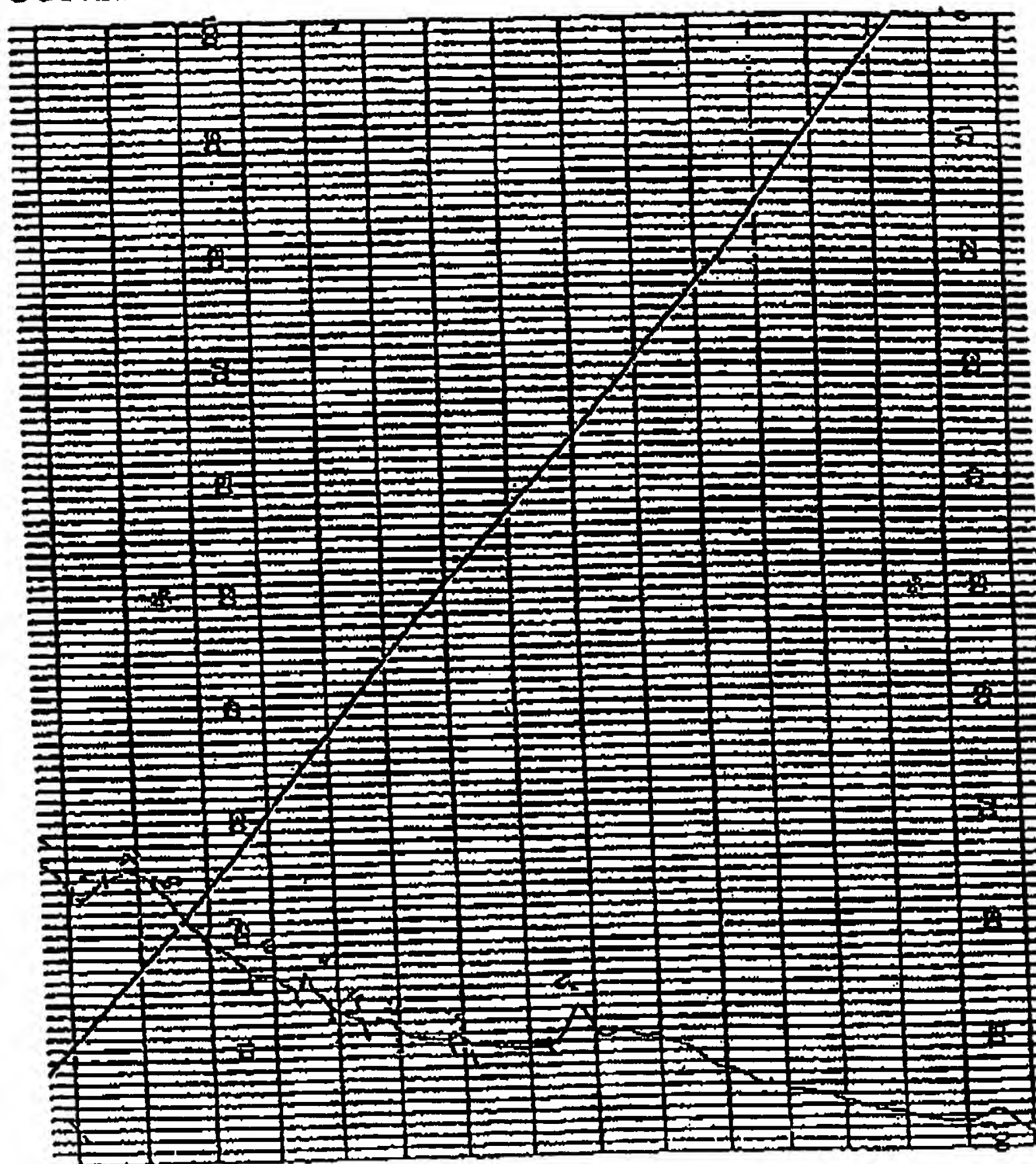


Fig. 4

**European Patent No 1 193 314**

**(Application No 01204340.2)**

**DANISCO A/S**

---

**Opposition by NOVOZYMES A/S**

---

TABLE OF CONTENTS

1. REQUESTS.....	1
2. STATEMENT OF GROUNDS AND EXTENT OF OPPOSITION .....	1
3. BACKGROUND.....	1
4. ADDED SUBJECT MATTER .....	2
5. THE PATENT IS INSUFFICIENT.....	3
6. THE PRIORITY CLAIM IS INVALID .....	5
7. CLAIM 1 LACKS NOVELTY OVER D4 .....	6
8. CLAIM 1 LACKS NOVELTY OVER D5.....	10
9. CLAIM 1 LACKS NOVELTY OVER D8.....	12
10. CLAIM 1 LACKS NOVELTY OVER D9 .....	12
11. CLAIM 1 LACKS NOVELTY OVER D10 .....	13
12. CLAIM 1 LACKS NOVELTY OVER D11 .....	14
13. CLAIM 1 LACKS NOVELTY OVER D12 .....	15
14. CLAIM 1 LACKS NOVELTY OVER D13 .....	15
15. CLAIM 1 LACKS AN INVENTIVE STEP OVER D10 .....	15
16. CLAIM 1 LACKS AN INVENTIVE STEP OVER D14 OR D15 .....	17
17. NO PREJUDICE AGAINST USING ENZYMES .....	18
18. CLAIM 2 ADDS NOTHING PATENTABLE TO CLAIM 1. .....	19
19. CLAIM 3 RENDERS THE PATENT INSUFFICIENT, LACKS NOVELTY OVER D4 AND LACKS AN INVENTIVE STEP .....	19
20. CLAIM 4 RENDERS THE PATENT INSUFFICIENT, LACKS NOVELTY OVER D4 AND LACKS AN INVENTIVE STEP .....	20
21. CLAIM 5 RENDERS THE PATENT INSUFFICIENT AND LACKS NOVELTY AND AN INVENTIVE STEP FOR THE SAME REASONS AS CLAIM 1 .....	21
22. CLAIM 6 LACKS NOVELTY AND LACKS AN INVENTIVE STEP FOR THE SAME REASONS AS CLAIM 1 .....	22
23. CLAIM 7 LACKS NOVELTY OVER D4 AND D5 .....	22
24. CLAIM 8 .....	22
25. SUMMARY .....	23
26. DOCUMENTS RELIED ON.....	24

Eric Potter Clarkson  
for opponent Novozymes A/S

## 1. REQUESTS

- 1.1 We request revocation of EP-B-1193314 ("the patent"), for all contracting states, and we request oral proceedings in the event that the Opposition Division is inclined to maintain the patent in any form.

## 2. STATEMENT OF GROUNDS AND EXTENT OF OPPOSITION

- 2.1 All claims of the patent are opposed. The opposition is based on Articles 100(a), 100(b) and 100(c) EPC.

## 3. BACKGROUND

- 3.1 The patent relates to the use of lipases in dough and baked products.
- 3.2 The application from which the patent is derived, European patent application no 01204340.2 ("the application"), is a divisional application based on European patent application no 98913539.7 ("the parent application"). The parent application was filed on 3 April 1998 and claims a priority date of 9 April 1997. The parent application was published as WO 98/45453 (filed herewith as D1).
- 3.3 The parent application granted as European patent no 0977869 with claims directed to uses of lipases derivable from *Aspergillus tubigenis* having specified activity and isoelectric point. A copy of EP-B-0977869 is filed herewith as D2.
- 3.4 In addition to the application, three other divisional applications have been filed by the proprietor. These applications are EP 040075818 (EP-A-1433852), EP 040075819 (EP-A-1466980) and EP 040076486.
- 3.5 EP-B-0977869, with its claims limited to lipases derivable from *Aspergillus tubigenis* and having a specified isoelectric point, has been opposed, but by another party.
- 3.6 The subject matter claimed in the originally filed application was clearly a single concept based on a specific lipase from *Aspergillus tubigenis*. The proprietor's actions in filing the application and three other divisional applications appears to be an attempt to create

Eric Potter Clarkson  
for opponent Novozymes A/S

uncertainty for third parties and to inadmissibly broaden the claims. This amounts to an abuse of the procedure for filing divisional applications.

#### 4. ADDED SUBJECT MATTER

4.1 The patent contains added subject matter for two reasons:

- (i) the parent application as originally filed was limited to lipases from *Aspergillus tubigenis*, whereas the claims in the patent are not so limited; and
- (ii) the parent application as originally filed was limited to lipases having an isoelectric point in the range of 3.5 to 4.5, as determined by isoelectric focusing, whereas the claims in the patent cover lipases having any isoelectric point.

4.2 The application was filed with claims that omitted the two features mentioned above and broadened the lipase to subject matter not disclosed in the parent application. Therefore, the patent fails to comply with Article 100(c) and Article 76(1) EPC.

4.3 In the parent application, as originally filed, Claim 1 was directed to a polypeptide that is derivable from *Aspergillus tubigenis* and that has an isoelectric point, as determined by isoelectric focusing, in the range of 3.5 to 4.5 (see page 82, lines 9 to 10 of D1). All of the other claims refer back to Claim 1, either directly or indirectly, and so also incorporate these two features. The summary of the invention on pages 5 and 6 of D1 is similarly limited, with the lipase first being defined in the same way as in Claim 1 and then being referred to as "the" polypeptide (i.e., having the same features).

4.4 The paragraph at page 10, lines 5 to 10 of D1 explains that the polypeptide can be derived from any organism (clearly intended to mean a host cell), but subject to the overriding requirement that it is also derivable from *Aspergillus tubigenis*. Thus, this paragraph does not provide any basis for a broader claim.

4.5 With regard to the isoelectric point of the polypeptide, the paragraph in the application as originally filed at page 7, lines 19 to 22 states that:

*"The polypeptide according to the invention has an isoelectric point determined by isoelectric focusing in the*

Eric Potter Clarkson  
for opponent Novozymes A/S

*range of 3.5-4.5 such as the range of 3.8 to 4.2 including an isoelectric point of 4.0 +/- 0.1" (emphasis added)*

- 4.6 Thus, the description confirms what is evident from the claims i.e., that the isoelectric point is not optional or preferred but is essential. The description states that the polypeptide "has" that isoelectric point (not that it "might" or "may have" that isoelectric point). This is completely consistent with the fact that this is presented as an essential feature in the claims. It is also consistent with the isoelectric point of the lipases exemplified (see page 24, lines 4 to 8 and page 50, lines 22 to 24).
- 4.7 It was the proprietor's decision to make the source of the polypeptide and its isoelectric point essential features and these cannot be omitted without adding subject matter. Indeed, the whole reason for prohibiting the addition of subject matter is to provide security for third parties against broad claims of this type that could not have been envisaged from the application as filed.
- 4.8 The omission of the essential feature of the isoelectric point of the polypeptide and the omission of the feature that the polypeptide is derivable from *Aspergillus tubigenis* therefore adds subject matter.
- 4.9 Accordingly, the patent must be revoked under Article 100(c) EPC.

## 5. THE PATENT IS INSUFFICIENT

- 5.1 It has been explained above in point 4 that the patent contains added subject matter. However, a further consequence of the proprietor's broadening amendment is that the patent is insufficient.
- 5.2 The disclosure in the application as originally filed is limited to the lipase from *Aspergillus tubigenis*. The claims in the patent as granted purport to cover lipases that are derivable from any source.
- 5.3 The absence from the specification of any disclosure of how to put the invention into effect with a lipase derivable from another source leads inevitably to two possibilities. Either lipases derivable from other sources were not available from the prior art, in which case the patent fails to disclose the claimed subject matter sufficiently to allow it to be carried out with anything other than the lipase from *Aspergillus tubigenis*. Or lipases derivable from other sources were available from the prior art, in which case the patent may be

Eric Potter Clarkson  
for opponent Novozymes A/S



sufficient but lacks novelty or lacks an inventive step (for the reasons set out below). The proprietor cannot have it both ways.

5.4 We believe that the patent is clearly lacking in inventive step for the reasons given below. However, in the event that the proprietor argues that the lipases of the prior art are not suitable for use in baking, then the patent must be insufficient due to the scope of Claim 1 and the inability of the person skilled in the art to put the claimed subject matter into effect throughout the scope of the claim (see decision T409/91).

5.5 We note that the proprietor seems to approve this approach to Article 83 EPC. In the proprietor's notice of opposition to European Patent No 0575133, the proprietor states that:

*"In order to obtain [the enzyme in question], from organisms other than the specific strains ... taught in [the patent] a skilled person would need to commence a huge scientific research project"*

and

*"This leads to an undue experimental burden on a skilled person. Thus, [the patent] does not disclose the invention in a manner which is sufficiently clear and complete for it to be carried out by a person skilled in the art over the whole of the broad field claimed".*

5.6 A copy of the notice of opposition is filed herewith as A1 and the relevant passage is in the paragraph bridging pages 1 and 2.

5.7 Certainly, if the proprietor believes that the known lipases are unsuitable for use according to Claim 1 in the patent, then the scope of Claim 1 means that the patent is insufficient because the scope of the claim completely fails to reflect the contribution to the art (see T694/92). At the most, the only contribution to the art could be the lipase from *Aspergillus tubigenis*.

5.8 Therefore, it appears that the patent fails to comply with Article 83 EPC and must be revoked under Article 100(b) EPC.

5.9 Further reasons why the patent is insufficient result specifically from the features of Claims 3, 4 and 5 and are set out below in connection with the discussion of those claims.

Eric Potter Clarkson  
for opponent Novozymes A/S

**6. THE PRIORITY CLAIM IS INVALID**

- 6.1 Before assessing the validity of the patent on the grounds of novelty (Article 54 EPC) and inventive step (Article 56 EPC), the validity of the claim to priority must be assessed. It is readily apparent that the claim to priority in the patent is invalid.
- 6.2 A copy of the priority document (DK 0400/97) is filed herewith as D3.
- 6.3 The disclosure in D3 is limited to polypeptides specifically identified by the three sequences in SEQ ID Nos 1, 2 and 3. This is evident from the claims in D3 (see Claim 1 on page 34; all of the other claims refer to Claim 1 either directly or indirectly). The statement of invention on page 3 of D3 is consistent with this.
- 6.4 D3 does not disclose a number of the features of Claim 1 in the patent.
- 6.5 Claim 1 requires that the lipase is capable of splitting off fatty acids having short, medium and long chain length. This feature is absent from D3.
- 6.6 Claim 1 specifies that the polypeptide retains at least 80 % activity after 4 days at 20°C and at a pH in the range of 3.5-8. This feature is also not disclosed in D3.
- 6.7 Claim 1 specifies that the polypeptide is capable of hydrolysing at least 10 % of the galactosyl diglycerides normally present in flour dough to galactosyl monoglycerides. Again, this feature is not disclosed in D3.
- 6.8 Additionally, the priority document D3 is limited to the use of the polypeptide in fat-free doughs (see, for example, page 3, lines 31, 36 and 47). Claims 1 to 5, 7 and 8 in the patent are not limited to use of the polypeptide in fat-free doughs.
- 6.9 The principle set out in Enlarged Board of Appeal decision G2/98 is that a claim to priority is only valid if the claimed subject matter is derivable directly and unambiguously from the priority document. There is no clear and unambiguous disclosure in D3 of at least the three features of Claim 1 identified above or the possibility of using anything other than a fat-free dough.

Eric Potter Clarkson  
for opponent Novozymes A/S

- 6.10 Accordingly, the claim to priority for Claim 1 is invalid. Since all of the claims in the patent are dependent on Claim 1, there is no valid claim to priority.
- 6.11 The relevant date for assessing whether or not a document qualifies as prior art under Article 54(2) or Article 54(3) EPC is therefore the filing date of 3 April 1998.

## 7. CLAIM 1 LACKS NOVELTY OVER D4

7.1 Claim 1 lacks novelty under Article 54 EPC over the disclosure in WO 94/04035 (Novo Nordisk A/S) (D4). D4 was published on 3 March 1994 and therefore qualifies as prior art for the patent under Article 54(2) EPC.

7.2 Claim 1 contains following features:

- (i) it is directed to the use of a polypeptide having lipase activity in the preparation of dough and/or baked product;
- (ii) the polypeptide is a triacylglycerol hydrolysing enzyme;
- (iii) the polypeptide is capable of splitting off short, medium and long chain fatty acids;

characterised in that

- (iv) the polypeptide is capable of hydrolysing galactolipids that are normally present in the flour to the corresponding galactosyl monoglycerides;
- (v) the polypeptide retains at least 80% activity after 4 days at 20 °C and a pH in the range of 3.5-8;
- (vi) the polypeptide is capable of hydrolysing at least 10 % of the galactosyl diglycerides normally present in flour dough to the corresponding monoglycerides; and
- (vii) the enzyme imparts improved pore homogeneity or reduced pore diameter to the product.

Eric Potter Clarkson  
for opponent Novozymes A/S

It is apparent that features (iv) and (vi) are linked. Feature (vi) merely quantifies feature (iv). Accordingly, these features are sometimes discussed together in the comments below.

- 7.3 We note, in passing, that (vii) is merely an inherent property of the enzyme – this is not the purpose of the use claim. The only use that is required of the enzyme is in the preparation of dough or baked product, apparently for any purpose. However, this is somewhat academic since feature (vii) is, in any case, disclosed in the prior art.
- 7.4 D4 relates to the use of lipases for improving the properties of dough or a baked product (see line 1 of the Abstract). This is feature (i) of Claim 1.
- 7.5 The lipases of D4 all have lipase activity as defined by LU (Lipase Units). LU are based on the hydrolysis of glycerin tributyrate (also known as tributyrin) as substrate (see D4 at page 15, lines 29 to 37). Tributyrin is a triacylglycerol. Therefore, the lipases of D4 have feature (ii) of Claim 1.
- 7.6 The lipases of D4 impart improved crumb structure to the bread (see page 6, line 1). Crumb structure is defined in terms of uniformity in D4 (see page 18, lines 26 to 31) by ranking a uniform structure higher than a non-uniform structure. This means homogeneity of the pores. This is confirmed by paragraph [0065] in the patent, which equates uniformity of crumb structure with homogeneity of the pores (see page 6, line 48 in particular). Thus, feature (vii) of Claim 1 is disclosed in D4.
- 7.7 Enzymes disclosed and exemplified in D4 include *Humicola lanuginosa* lipase (see Example 2) and *Rhizomucor miehei* lipase (see Example 3). The opponent, Novozymes A/S, is the direct successor in title of Novo Nordisk A/S insofar as D4 and the enzyme business in general are concerned. Therefore, the opponent is ideally placed to know about the disclosure in D4.
- 7.8 Functional features (iii), (iv), (v) and (vi) are not explicitly disclosed in D4. However, experiments carried out by the opponent show that these properties are properties of the lipases of D4.
- 7.9 We are enclosing two experimental reports that have been prepared by the opponent. The first report (E1) shows the baking performance and the activity on galactolipids of four lipases of the prior art. The second report (E2) is an analysis of the stability of the lipases and

Eric Potter Clarkson  
for opponent Novozymes A/S

their ability to split off short, medium and long chain fatty acids from glycerides.

- 7.10 E1 contains the results of baking trials carried out according to the disclosure in the patent, as far as this can be determined from the patent specification. Similarly, the results in E2 correspond to the tests given in the patent, as far as it is possible to determine these from the specification.
- 7.11 E1 shows that the *Humicola lanuginosa* lipase and the *Rhizomucor miehei* lipase of D4 have features (iv) and (vi) as well as confirming that they have feature (vii), as D4 already discloses.
- 7.12 In E1, baking trials were carried out using the lipase from *Humicola lanuginosa* (now also known as *Thermomyces lanuginosus*). Table 1 on page 2 of E1 shows that the lipase from *Humicola lanuginosa* was expressed in *Aspergillus oryzae*, in exactly the same way as in D4 (see D4 at page 14, lines 31 to 33). Experiments were carried out using from 1050 to 39,450 LU/kg flour. This is completely in line with the disclosure in D4; the dosage of lipase disclosed in D4 is up to 100,000 LU/kg, with up to 50,000 LU/kg being preferred (see page 8, lines 5 to 7 and line 15) and dosages of 250 to 50,000 LU/kg are used in Table 3 on page 21 of D4.
- 7.13 As is evident from Table 2 on page 4 of E1, the use of lipase from *Humicola lanuginosa* in the baking trials gave an increase in the uniformity of the pore size at dosages of 400, 1,140, 1,900, 3,800, 5,000 and 7,600 LUS/kg (LUS are the lipase units used in the patent) or 1,050, 3,000, 5,000, 10,000, 13,150 and 20,000 LU/kg, respectively. Also, at all of the dosages tested, the pore diameter was reduced. This is evident from the second to ninth rows and the second to fourth columns in Table 2 on page 4 of E1.
- 7.14 Therefore, E1 illustrates the fact that the disclosure in D4 is a disclosure of feature (vii) of Claim 1.
- 7.15 Additionally, E1 shows that the lipase from *Humicola lanuginosa* of D4 has features (iv) and (vi) of Claim 1 i.e., that the polypeptide is capable of hydrolysing at least 10 % of the galactosyl diglycerides normally present in flour dough to the corresponding monoglycerides. This fact is evident from the last two columns of Table 2 on page 9 of E1. It is not clear from the patent as to how the basis for the 10 % figure for the hydrolysis of the galactosyl diglycerides should be determined. The figures in E1 are given in both molar percentages (see the second to last column in Table 2)

Eric Potter Clarkson  
for opponent Novozymes A/S

and in percentages by weight (see the last column in Table 2). It is evident that regardless of how the percentage is calculated, the lipases of D4 are capable of hydrolysing at least 10 % of the galactosyl diglycerides normally present in flour dough to the corresponding monoglycerides as required by feature (vi) of Claim 1. Thus, the *Humicola lanuginosa* lipase of D4 has features (iv) and (vi) of Claim 1.

- 7.16 The results in E1 also show that the lipase from *Rhizomucor miehei* that is disclosed in D4 has features (iv) and (vi) of Claim 1 as well as confirming that it has feature (vii). The *Rhizomucor miehei* lipase used in D4 was expressed in *Aspergillus oryzae*, in the same way as in D4 (see D4 at page 14, lines 35 to 39).
- 7.17 Thus, Table 2 on page 9 of E1 shows that the *Rhizomucor miehei* lipase increases pore uniformity and decreases pore diameter (see the last three rows, second to fourth columns) and that it hydrolyses at least 10 % (by weight or by moles) of the digalactosyl diglycerides in flour to the corresponding monoglycerides (see the last two columns in Table 2).
- 7.18 Thus, the only remaining features of Claim 1 are features (iii) and (v) relating to the ability of the lipases of D4 to hydrolyse short, medium and long chain fatty acids and to the stability of the lipases.
- 7.19 E2 shows that the *Humicola lanuginosa* lipase and the *Rhizomucor miehei* lipase of D4 have features (iii) and (v) of Claim 1. Thus, Table 2 on page 2 of E2 shows that the two lipases retain more than 80 % activity after 4 days at 20 °C and at pH 3.5, 5.5 and 8.0. Figure 1 on page 3 of E2 shows that both enzymes are capable of splitting off short, medium and long chain fatty acids.
- 7.20 Therefore, since all of the features (i) to (vii) are disclosed in D4, either directly or inherently, Claim 1 lacks novelty over D4.
- 7.21 As an aside, we note that Example 12 on page 31 of the patent presents data purporting to show that two lipases of the prior art, namely 1885 and 1787, have negligible effect on the hydrolysis of digalactosyl diglyceride (DGDG). 1885 is Novozym 677 BG (see paragraph [0207] for an identification of the enzymes), a product produced by the opponent, and is the lipase of D4 from *Humicola lanuginosa*.
- 7.22 However, Example 12 in the patent is a completely unfair comparison and does not reflect the true teaching in D4.

Eric Potter Clarkson  
for opponent Novozymes A/S



7.23 Example 12 uses very different dosages for the three enzymes. The dosages (in the same units, LUS/kg of flour) are as follows:

1885 – 400 and 800  
1757 – 1770 and 2360  
Lipase 3 – 10,000 and 20,000

7.24 This is not only a completely unfair comparison (because at least 4 to 50 times more of the Lipase 3 was added), it also does not correspond to the teaching in D4. For instance, when the corresponding lipase from *Humicola lanuginosa* is used in D4, the dosage of lipase is up to 100,000 LU/kg, with up to 50,000 LU/kg being preferred (see page 8, lines 5 to 7 and line 15).

7.25 Example 12 in the patent is thus no evidence at all for a distinction over the lipases of the prior art.

## 8. CLAIM 1 LACKS NOVELTY OVER D5

8.1 Claim 1 lacks novelty over EP-A-0869167 (Novo Nordisk A/S) (D5). D5 was published on 7 October 1998. However, since the filing date and all of the priority dates of D5 are before the effective date of the patent of 3 April 1998, D5 qualifies as prior art for the patent under Article 54(3) EPC.

8.2 D5 discloses a phospholipase from *Fusarium oxysporum* (see page 10, line 34). The enzyme has lipase activity (see Table 7 on page 31 and page 22, line 9), as defined by LU i.e., activity on tributyrin (see page 29, lines 45 to 47). Therefore, the enzyme of D5 has feature (ii) of Claim 1.

8.3 The enzyme of D5 is disclosed for use in baking (see page 21, line 11 to page 22, line 5 and Examples 20 and 21 on pages 46 to 49). This is feature (i) of Claim 1.

8.4 The enzyme of D5 improves crumb structure (see page 21, line 26). This is understood by those skilled in the art to mean an improvement in pore homogeneity, as D4 illustrates. Thus, the enzyme of D5 has feature (vii) of Claim 1.

8.5 Like D4, D5 does not explicitly disclose features (iii) to (vi) of Claim 1 in the patent. However, according to the proprietor, D5 has

Eric Potter Clarkson  
for opponent Novozymes A/S

features (iv) and (vi) and this is evident from the proprietor's own disclosure in the later filed EP 1108360 (D6).

- 8.6 D6 relates to a method of improving dough and baked products (see the title and page 3, lines 47 to 48) by adding an enzyme capable of hydrolysing lipids (i.e., a lipase; see also paragraph [0028]) to the dough (page 3, line 56 to page 4, line 1). According to D6, a preferred enzyme is Lipase SP972 (see paragraph [0037]). As explained in paragraph [0174], Lipase SP972 was obtained from Novo Nordisk (predecessor of Novozymes A/S) and was given that designation by Novo Nordisk.
- 8.7 The Declaration of Kim Borch filed herewith as D7 confirms that sample SP972 is the *Fusarium oxysporum* phospholipase of D5.
- 8.8 According to what the proprietor states in D6, SP972 gave a more homogenous crumb structure when used in baking (see paragraph [0212] in D6). This is feature (vii) of Claim 1 in the patent.
- 8.9 Table 2.3 on page 20 of D6 shows that the results of the proprietor's tests are that SP972 (used in Test dough 5) reduces the content of digalactosyl diglyceride (DGDG) from 0.209 to 0.022 and that the digalactosyl monoglyceride content of the dough is increased from 0.015 to 0.103, accordingly. These are features (iv) and (vi) of Claim 1.
- 8.10 According to the results obtained by the proprietor in Table 10.3 on page 30 of D6, SP972 is capable of hydrolysing medium and long chain fatty acids and its activity on tributyrin (mentioned above) means that it has activity on short chain fatty acids. This is feature (iii) of Claim 1.
- 8.11 This leaves only the functional feature (v) relating to the stability of the enzyme.
- 8.12 E2 shows that the *Fusarium oxysporum* lipase has feature (v). Thus, Table 2 on page 2 of E2 shows that the lipase retains at least 88 % activity after 4 days at 20 °C and at pH 3.5, 5.5 and 8.0.
- 8.13 Therefore, since all of features (i) to (vii) are disclosed in D5, either directly or implicitly, Claim 1 lacks novelty over D5.
- 8.14 For the avoidance of any doubt, we wish to point out that D5 is not being combined with any other document to show a lack of novelty. D6 and D7 are simply cited to show the proprietor's view that the

Eric Potter Clarkson  
for opponent Novozymes A/S

enzyme of D5 has many of the functional properties specified in Claim 1 of the patent and E2 is cited to show that the enzyme of D5 also has the remaining features in Claim 1.

9. **CLAIM 1 LACKS NOVELTY OVER D8**

9.1 Claim 1 also lacks novelty over WO 98/31790 (Rohm GmbH) (D8). D8 was published on 23 July 1998 and has a filing date of 8 January 1998. D8 entered the European regional phase as EP-A-0904357. A printout of the EPO register entry for D8 is enclosed as D8A. Therefore, D8 is citable against the patent under Article 54(3) EPC.

9.2 D8 discloses in Example 6 the use of an enzyme in baking to give a more regular pore structure. The enzyme of D8 from *Aspergillus foetidus* differs from the enzyme of the patent by a single amino acid residue (compare SEQ ID No 9 in the patent with SEQ ID No 2 in D8). The only difference is a replacement of a valine residue (V) by a leucine residue (L) at position 256. Both V and L have aliphatic side chains and would be expected to have substantially the same properties.

9.3 It is reasonable to expect that the enzyme of D8 will have substantially the same properties as the enzyme of the patent. If the proprietor wishes to argue that the 0.4 % difference in amino acid sequence and the closely similar structure of the different amino acid residue have any effect on the properties of the enzyme, then the onus must be with the proprietor to provide evidence of such an alleged effect.

9.4 Therefore, Claim 1 lacks novelty over D8.

10. **CLAIM 1 LACKS NOVELTY OVER D9**

10.1 Claim 1 also lacks novelty over the disclosure in Egypt J Food Sci, 14, No 1, pp175-182, (1986) (D9). D9 forms part of the state of the art for the patent under Article 54(2) EPC.

10.2 D9 discloses the use of lipase from *Rhizopus delemar* in bread making (see the Title). The lipase can hydrolyse triacylglycerols (see Table 1 on page 177 of D9). These are features (i) and (ii) of Claim 1.

Eric Potter Clarkson  
for opponent Novozymes A/S

- 10.3 The lipase of D9 improves crumb texture (see Table 2 on page 178). This is feature (vii) of Claim 1.
- 10.4 The fact that the lipase from *Rhizopus delemar* reduces pore diameter and increases pore homogeneity is also evident from E1. Thus, Table 2 on page 9 of E1 shows that this lipase increased pore homogeneity (uniformity) and reduced pore diameter.
- 10.5 The lipase of D9 also has features (iii), (iv), (v) and (vi) of Claim 1. The lipase is capable of splitting off short, medium and long chain fatty acids (see Figure 1 on page 3 of E2), it is capable of hydrolysing at least 10 % of the galactosyl diglycerides normally present in flour dough to the corresponding monoglycerides (see Table 2 on page 4 of E1) and it retains at least 80% activity after 4 days at 20 °C and a pH in the range of 3.5-8 (see Table 2 on page 2 of E2). In connection with this latter feature, we note that there is no requirement in Claim 1 for stability throughout this pH range and apparently stability at one pH value within this range is enough to satisfy the claim (see, for example, Claim 2, which narrows this range).
- 10.6 Therefore, Claim 1 lacks novelty over D9.

## 11. CLAIM 1 LACKS NOVELTY OVER D10

- 11.1 Claim 1 lacks novelty over the document that was brought to the attention of the examiner during examination of the application i.e., Poulsen et al, "Effect And Functionality Of Lipases In Dough And Bread", The first European Symposium on Enzymes and Grain Processing, TNO Nutrition and Food Research Institute, 1997, ISBN 9075202040 (D10). D10 is the Proceedings of ESEGP-1, Noordwijkerhout, The Netherlands, December 2-4, 1996. This is evident from the two front sheets of the document, as filed by the proprietor during examination, a copy of which is enclosed as D10A.
- 11.2 A clearer copy of D10 is filed herewith.
- 11.3 In D10, the lipase used in all of the experiments, except for Figure 4, was the same and was Grindamyl EXEL 16 (see page 207, middle of the page, under the heading "Lipase preparation"). The lipase that was used in Figure 4 is not identified.
- 11.4 The disclosure of Grindamyl EXEL 16 in D10 destroys the novelty of Claim 1 in the patent.

Eric Potter Clarkson  
for opponent Novozymes A/S

- 11.5 Grindamyl EXEL 16 is the lipase from *Humicola lanuginosa* disclosed in D4. This has been acknowledged by the proprietor in the later published WO 03/099016 (Danisco A/S) (D16). Page 29, lines 23 to 24 of D16 confirms that Grindamyl EXEL 16 is the lipase from *Thermomyces lanuginosa* (formerly *Humicola lanuginosa*) expressed in *Aspergillus oryzae*.
- 11.6 As explained in section 7 above, the lipase from *Thermomyces lanuginosa* (formerly *Humicola lanuginosa*) has features (iii), (iv), (v), (vi) and (vii) of Claim 1 in the patent.
- 11.7 D10 relates to the use of the lipases in dough and bread (see the Title). The lipase hydrolyses triacylglycerols (see page 207 under the heading "Lipase Preparation" and Table 1 on page 211). These are features (i) and (ii) of Claim 1.
- 11.8 The effect of the lipase in D10 is an increase in pore homogeneity and a decrease in pore diameter in the dough. This is clear from Figure 5 and a comparison of the loaf on the left of the picture with the loaf second from the left. Hence, D10 even explicitly teaches feature (vii) of Claim 1.
- 11.9 Therefore, since all of the features of Claim 1 are disclosed in D10, Claim 1 lacks novelty over D10.

## 12. CLAIM 1 LACKS NOVELTY OVER D11

- 12.1 Claim 1 lacks novelty over Jensen, Bericht über die 48. Tagung für Bäckerei-Technologie und 3. Tagung für Konditorei-Technologie 1997, Vorträge, gehalten anlässlich der Tagung der Arbeitsgemeinschaft Getreideforschung e.V. vom 4. - 6. November 1997 - - Detmold: Granum-Verl., 1998. 167 S. : Ill.; graph. Darst. - (Veröffentlichungen der Arbeitsgemeinschaft Getreideforschung e.V. ; 274) (D11).
- 12.2 The disclosure in D11 is somewhat similar to that in D10.
- 12.3 Thus, D11 describes the use of the lipase Grindamyl EXEL 16 in baking to improve crumb structure (see point 3.3 on page 70) and shows the effects of the lipase, which are increased pore homogeneity and reduced pore diameter, in Figures 5 to 7 on pages 75 and 76. As explained above, Grindamyl EXEL 16 is the lipase from *Humicola lanuginosa*.

Eric Potter Clarkson  
for opponent Novozymes A/S

- 12.4 Therefore, for essentially the same reasons as given in relation to D10, Claim 1 lacks novelty over D11.

**13. CLAIM 1 LACKS NOVELTY OVER D12**

- 13.1 JP-A-55-131340 (D12) discloses the use of lipases in baking. D12A is an abstract of D12. In Examples 1 and 4, D12 describes the use of lipases from *Rhizopus delemar* and *Candida cylindracea*, respectively.
- 13.2 For essentially the reasons given above in relation to D9, Claim 1 lacks novelty over D12.

**14. CLAIM 1 LACKS NOVELTY OVER D13**

- 14.1 Another disclosure of the use of a lipase, having all of the features of Claim 1, in baking can be found in GB 1086550 (R T Vanderbilt Company, Inc) (D13), which was published in 1967. D13 discloses a lipase from *Candida cylindracea* (see page 3, lines 8 to 10) for use in dough and baked products (see the title). The lipase of D13 is believed to fulfil the stability feature of Claim 1 in the patent.
- 14.2 Therefore, Claim 1 lacks novelty over D13.

**15. CLAIM 1 LACKS AN INVENTIVE STEP OVER D10**

- 15.1 Insofar as it teaches the use in baking of lipases other than Grindamyl EXEL 16, the disclosure in D10 renders the claimed subject matter obvious.
- 15.2 As explained in section 11 above, D10 discloses the use of lipases in general in dough and bread. These are features (i) and (ii) of Claim 1.
- 15.3 D10 states that the lipase used in Figure 4 is not the same as that used elsewhere in the document. However, no distinction is made in D10 between different lipases and the teaching of the document is that these properties are generally shared by all lipases used in baking. Indeed, D10 postulates on pages 205 to 206 in points 1 to 4 and on pages 206 to 207 in points 1 to 6 that lipases work by forming polar lipids, which produce a stable foam. This mechanism is taught by D10 as being common to all lipases.

Eric Potter Clarkson  
for opponent Novozymes A/S



- 15.4 D10 also makes it clear that the positive effects of adding lipases to dough (points 1 and 4 to 6 on pages 206-7) outweigh the negative effects, particularly the known negative effect of free fatty acids in dough (points 2-3 on page 207). Indeed, this must be correct, otherwise there would have been no teaching in the art about using lipases in baking. There cannot be any prejudice against using lipases given the huge number of documents (including D4 to D13) advocating the use of lipases in baking and also the availability of lipases sold for use in baking before the filing date, such as Grindamyl EXEL 16. The conclusions on page 213 of D10 give a strong message about the benefits of adding lipase to dough, serving to confirm that there was no prejudice against adding lipases to dough.
- 15.5 Figure 4 on page 211 of D10 shows that the lipase hydrolyses DGDG to DGMG. Thus, the peak for DGDG at about 12.73 min decreases substantially (apparently by far more than 10 %), whilst that for DGMG increases substantially; this is evident from a comparison of the HPLC chromatograms for the control and lipase treated samples in Figure 4. These are features (iv) and (vi) of Claim 1.
- 15.6 The only features of Claim 1 that are not specifically disclosed in D10 are: feature (iii) that the polypeptide is capable of splitting off short, medium and long chain fatty acids; and feature (v) that the polypeptide retains at least 80% activity after 4 days at 20 °C and a pH in the range of 3.5-8.
- 15.7 Features (iii) and (v) represent arbitrary features of the specific *Aspergillus tubigenis* lipase exemplified in the patent and do not contribute at all to the solution to the technical problem as set out in the patent. Accordingly, they must be disregarded when assessing inventive step. As set out by the Board of Appeal in T285/91, when assessing inventive step, only those features that contribute causally to the solution to the problem are to be considered. Features which do not contribute to the solution to the problem are to be disregarded (T37/82).
- 15.8 However, if the features are taken into account, then the objective problem based on D10 is merely how to select a suitably stable enzyme.
- 15.9 The solution, which is lipases having the stability as defined in feature (v), is immediately evident to the skilled person from D4, D5,

Eric Potter Clarkson  
for opponent Novozymes A/S

17

D6 and D9, all of which disclose enzymes for use in baking having the specified stability, as E2 shows.

15.10 The conditions for the stability feature (v) (4 days at 20 °C) clearly relate to the storage stability of the lipase and are unrelated to the conditions under which the lipase is active in dough. There can be nothing inventive in selecting an enzyme that has this storage stability when this is clearly an obvious desideratum and, in any case, was already a feature of known lipases used in baking.

15.11 Therefore, the claimed subject matter lacks an inventive step under Article 56 EPC over D10, either alone or in combination with any one of D4, D5, D6 or D9.

**16. CLAIM 1 LACKS AN INVENTIVE STEP OVER D14 OR D15**

16.1 Claim 1 lacks an inventive step over Marion *et al* in Wheat Structure, Biochemistry and Functionality, "Wheat Lipids and Lipid-Binding Proteins: Structure and Function", The Royal Society of Chemistry, Proceedings of a conference organised by the Royal Society of Chemistry Food Chemistry Group, held on 10-12 April 1995, Reading, UK (D14) or Marion *et al*, Interactions: The Keys to Cereal Quality, published by the American Society of Cereal Chemists on March 4, 1998 (D15). Also enclosed is a letter from the publishers of D15 confirming the publication date (D15A). Both D14 and D15 form part of the state of the art for the patent under Article 54(2) EPC.

16.2 D14 teaches at page 257, last full paragraph, that, for modifying lipid structure:

*"lipases are good candidates because they are able to generate polar lipids – monoglycerides – from non polar triglycerides and detergent like molecules from phospholipids and glycolipids (lysophospholipids, galactosylmonoglycerides) which are good improvers of bread volume and texture"* (emphasis added)

16.3 Thus, D14 teaches the skilled person to use lipases that hydrolyse triacylglycerols and galactosyl diglycerides in order to improve the texture of bread.

Eric Potter Clarkson  
for opponent Novozymes A/S

- 16.4 D14 does not explicitly disclose the activity of the enzyme, its ability to split off short, medium and long chain fatty acids or the actual degree of hydrolysis of the galactosyl diglycerides. However, as mentioned above and as acknowledged by the proprietor in the two part form of the claim, the ability to hydrolyse short, medium and long chain fatty acyl groups in glycerides is simply an inherent feature of lipases conventionally used in baking and should be disregarded since it does not contribute to solving any technical problem. Similarly, the degree of hydrolysis of the galactosyl diglycerides is inherent in the lipases of the prior art. Even if this were not inherent, it would be an obvious desideratum given the teaching in D14 and, accordingly, could not involve an inventive step.
- 16.5 If D14 is taken as the closest prior art, then the problem can again be seen to be how to provide a stable enzyme. In the same way as for the analysis based on D10 as closest prior art, the solution, which is lipases having the required stability as defined in feature (v), is immediately evident to the skilled person from D4, D5, D6 and D9; all of which disclose lipases for use in baking having the required stability.
- 16.6 Therefore, the subject matter of Claim 1 lacks an inventive step over D14, alone or in combination with any one of D4, D5, D6 and D9.
- 16.7 A similar teaching to that in D14 can be found in D15 in the paragraph bridging pages 157 and 158. Therefore, Claim 1 also lacks an inventive step over D15, alone or in combination with any one of D4, D5, D6 and D9.

## 17. NO PREJUDICE AGAINST USING ENZYMES

- 17.1 D14 states that the drawback of enzymes is that they generate free fatty acids. However, this is not an obstacle for a person skilled in the art or any prejudice against using enzymes for at least three reasons.
- 17.2 Firstly, D14 overrides this teaching by stating in the same paragraph on page 257 that:

*"The best way of modifying lipid structure is certainly through the use of enzymes and especially hydrolytic enzymes. In this regard lipases are good candidates..."*

Eric Potter Clarkson  
for opponent Novozymes A/S

- 17.3 Secondly, D10 teaches in point 3 on page 207 that the positive effects of adding lipases to dough completely outweigh the negative effects of free fatty acids in the dough (see point 15.4 above).
- 17.4 Thirdly, as shown by all of the prior art documents mentioned above, there was, in fact, teaching towards using lipases in baking. Indeed, the proprietor even sold the lipase Grindamyl EXEL 16 for use in baking well before the priority date and effectively recommended its use in D10. (Again, see the comments in point 15.4 above.)
- 17.5 All three of the above points are illustrations of the common general knowledge among those skilled in the baking art that the addition of lipases to dough is beneficial.
- 17.6 Therefore, there was no prejudice in the art away from using enzymes for this purpose.

**18. CLAIM 2 ADDS NOTHING PATENTABLE TO CLAIM 1**

- 18.1 Claim 2 adds to Claim 1 the feature that the pH is in the range of 5-7.
- 18.2 Since the enzymes of D4, D5, D8 and D9 have the specified activity in this pH range, as E2 shows in its testing of the enzyme at pH 5.5 in Table 2 on page 2, Claim 2 adds nothing patentable to Claim 1.
- 18.3 Moreover, a pH of 5-7 is common for dough, so this would be the most obvious pH range in which to maintain the stability of the lipase and to determine stability. Accordingly, Claim 2 is no more inventive than Claim 1.
- 18.4 Thus, Claim 2 lacks novelty or lacks an inventive step for the same reasons as Claim 1.

**19. CLAIM 3 RENDERS THE PATENT INSUFFICIENT, LACKS NOVELTY OVER D4 AND LACKS AN INVENTIVE STEP**

- 19.1 Claim 3 adds to Claims 1 and 2 a first feature that the polypeptide retains at least 60 % of its activity after 1 hour at 60 °C in 100 mM sodium acetate buffer at pH 5.0. The claim then states a further second feature that it includes a polypeptide that retains at least 80 % of its activity after 1 hour at 50 °C under the same conditions.

Eric Potter Clarkson  
for opponent Novozymes A/S

19.2 As a first point, the claim is insufficient under Article 83 EPC because the two different first and second functional features specified in Claim 1 can be overlapping but the second feature that is "included" is not a subset of the first feature. The skilled person is unable to determine whether certain polypeptides fall within the scope of the claim. For example, the skilled person cannot determine whether a polypeptide that retains at least 80 % of its activity after 1 hour at 50 °C under the specified conditions (thus satisfying the first feature) but which does not retain at least 60 % of its activity after 1 hour at 60 °C, is inside or outside the claim. The skilled person is thus unable to determine whether or not he or she is working within the area of the claims (see T256/87). The patent must therefore be revoked under Article 100(b) EPC.

19.3 As a second point, Claim 3 fails to add anything patentable to Claim 1. As shown by E2 in Table 3 on page 3, the lipase from *Humicola lanuginosa* of D4 has both the first and second features of Claim 3.

19.4 Claim 3 also lacks an inventive step over D10 or D14 or D15. Claim 3 specifies an activity for the enzyme, which is simply an obvious desideratum that cannot confer patentability on the claim.

19.5 The temperatures specified in Claim 3 would typically only be encountered, very briefly, during the actual baking of the dough. However, since the proprietor has not shown that the lipase needs to be active during baking or that lipase activity during baking has any effect, the two features of Claim 3 merely represent arbitrary limitations that do not solve any technical problem and cannot contribute to an inventive step.

**20. CLAIM 4 RENDERS THE PATENT INSUFFICIENT, LACKS NOVELTY OVER D4 AND LACKS AN INVENTIVE STEP**

20.1 Claim 4 adds to Claims 1 to 3 the feature that when the polypeptide is added to bread dough in an amount of 5,000 lipase units per kg of flour, the average pore diameter of the crumb of the bread made from the dough is reduced by at least 10 %.

20.2 As is the case for Claim 3, the skilled person is unable to determine whether or not a given polypeptide is within the scope of Claim 4 and, therefore, the patent does not comply with Article 83 EPC insofar as Claim 4 is concerned.

Eric Potter Clarkson  
for opponent Novozymes A/S



- 20.3 Claim 4 refers to "lipase units". In the description at page 8, line 17 to page 9, line 13, five different methods are given for determining lipase activity using four different substrates.
- 20.4 Moreover, the method of determining the percentage reduction in pore diameter is not described in the patent. There is no indication as to how this is measured or how the bread is prepared. The measurements depend on factors that are simply not described in the patent.
- 20.5 For both of the reasons in points 20.3 and 20.4 above, Claim 4 renders the patent insufficient.
- 20.6 Additionally, even if the lipase units in Claim 4 are sufficiently disclosed (which we do not accept), then it is clear from E1 (see the third to last column in Table 2 of E1) that at least the *Rhizomucor miehei* lipase of D4 possesses this feature. Therefore, Claim 4 lacks novelty over D4.
- 20.7 The feature of Claim 4 is clearly an obvious desideratum; decreased pore diameter is an obvious goal in the context of baking. This fact is evident from D4 and D10, for example. This feature cannot, therefore, confer patentability on the already obvious use of Claim 1 and, accordingly, Claim 4 lacks an inventive step.

**21. CLAIM 5 RENDERS THE PATENT INSUFFICIENT AND LACKS NOVELTY AND AN INVENTIVE STEP FOR THE SAME REASONS AS CLAIM 1**

- 21.1 The only difference between Claim 4 and Claim 5 is that the pore homogeneity of the crumb is increased by 5 %, rather than the pore diameter being reduced by at least 10 %.
- 21.2 Claim 5 therefore renders the patent insufficient for essentially the same reasons as Claim 4. For example, there is no indication for the skilled person as to how to determine what is meant by lipase units or how to select suitable conditions for the baking test.
- 21.3 The feature of Claim 5, as far as it can be ascertained, is shared by all of the lipases tested in E1. Therefore, Claim 5 lacks novelty for the same reasons as Claim 1.
- 21.4 Even if the feature of Claim 5 had not been possessed by the lipases of the prior art, it represents an obvious desideratum for the skilled

Eric Potter Clarkson  
for opponent Novozymes A/S



22

person and cannot confer an inventive step on the otherwise obvious claimed subject matter.

**22. CLAIM 6 LACKS NOVELTY AND LACKS AN INVENTIVE STEP FOR THE SAME REASONS AS CLAIM 1**

- 22.1 Claim 6 adds to the preceding claims the feature that the dough is a fat-free dough.
- 22.2 The dough of D4 is a fat-free dough (see page 3, lines 21 to 22). The doughs prepared in Examples 20 and 21 on pages 46 and 48 of D5 (see the "Basic Recipes") are also fat-free. In Example 6 of D8, the dough is fat-free. Also, in D9, the bread is fat-free (see point 3 on page 176).
- 22.3 Therefore, Claim 6 lacks novelty over D4, D5, D8 and D9 for the same reasons as Claim 1.
- 22.4 Claim 6 cannot involve an inventive step since using a fat-free dough with a lipase is absolutely conventional, as D4, D5, D8 and D9 illustrate.

**23. CLAIM 7 LACKS NOVELTY OVER D4 AND D5**

- 23.1 Claim 7 adds to the preceding claims the feature that the dough or the baked product comprises an emulsifier.
- 23.2 Emulsifiers are disclosed as being present in the dough together with lipases in D4 at page 11, lines 4 to 13. D5 describes emulsifiers as constituents of the dough at page 21, lines 45 to 47.
- 23.3 Therefore, Claim 7 lacks novelty over D4 and D5.
- 23.4 Claim 7 cannot involve an inventive step because the general use of emulsifiers in baking is absolutely routine. Adding emulsifiers to dough is a widespread and common global practice.

**24. CLAIM 8**

Eric Potter Clarkson  
for opponent Novozymes A/S

- 24.1 Claim 8 adds to Claims 1 to 7 the feature that the polypeptide is capable of hydrolysing at least 25 % of the galactosyl diglycerides normally present in the flour to galactosyl monoglycerides.
- 24.2 E1 shows that the lipases of D4 and D9 are capable of hydrolysing at least 25 % of the galactosyl diglycerides normally present in the flour to galactosyl monoglycerides (see Table 2 on page 4 of E1).
- 24.3 D6 shows on page 27 in Table 9.2 that SP 972 can hydrolyse DGDG from 0.204 wt % in a control dough down to a level of 0.026 wt %. This corresponds to a degree of hydrolysis of  $(0.204-0.026)/0.204$ , or about 87 %.
- 24.4 Therefore, Claim 8 lacks novelty over D4, D5 and D9.
- 24.5 The degree of hydrolysis specified in Claim 8 is an obvious desideratum given the teaching in D10, D14 and D15 of the usefulness of generating galactosyl monoglycerides in the dough. Thus, the subject matter of Claim 8 lacks an inventive step for the same reasons as Claim 1.

## 25. SUMMARY

- 25.1 The patent contains added subject matter because it omits the feature that the polypeptide has an isoelectric point of from 3.5 to 4.5 and is from *Aspergillus tubigenis*. Both of these features were presented as being essential in the application as originally filed. Therefore, Article 100(c) EPC prejudices maintenance of the patent as granted.
- 25.2 Either the patent is insufficient because it fails to disclose the claimed subject matter sufficiently to allow it to be carried out with anything other than lipase from *Aspergillus tubigenis* or lipases derivable from other sources were available from the prior art, in which case the patent may be sufficient but lacks novelty or lacks an inventive step.
- 25.3 The claim to priority is invalid because the priority document failed to disclose *inter alia* the 80 % stability feature in Claim 1.
- 25.4 Claim 1 lacks novelty under Articles 54 and 100(a) EPC over D4, D5, D8, D9, D10, D11, D12 and D13 which disclose the use of lipase enzymes in baking. The lipases have all of the features of Claim 1.

Eric Potter Clarkson  
for opponent Novozymes A/S

- 25.5 Claim 1 lacks an inventive step under Articles 56 and 100(a) EPC over D10, either alone or in combination with any one of D4, D5, D6 or D9. D4, D5, D6 and D9 provide the solution to the problem based on D10 of identifying a suitable lipase. Similarly, Claim 1 lacks an inventive step over D14 or D15, alone or in combination with any one of D4, D5, D6 and D9.
- 25.6 Claims 3, 4 and 5 render the patent insufficient under Articles 83 and 100(b) EPC because a person skilled in the art is unable to determine whether or not a given lipase is inside or outside the scope of the claims.
- 25.7 Claims 2 to 8 add nothing patentable over Claim 1 and lack novelty or lack an inventive step for the same reasons.

## 26. DOCUMENTS RELIED ON

- D1 WO 98/45453 (published PCT application)
- D2 EP-B-0977869 (parent patent)
- D3 DK 0400/97 (priority document)
- D4 WO 94/04035 (Novo Nordisk A/S)
- D5 EP-A-0869167 (Novo Nordisk A/S)
- D6 EP 1108360 (Danisco A/S)
- D7 Declaration of Kim Borch
- D8 WO 98/31790 (Rohm GmbH)
- D9 Egypt J Food Sci, 14, No 1, pp175-182, (1986)
- D10 Poulsen et al, "Effect And Functionality Of Lipases In Dough And Bread", The first European Symposium on Enzymes and Grain Processing, TNO Nutrition and Food Research Institute, 1997, ISBN 9075202040
- D10A Front two sheets of D10, as filed by the proprietor
- D11 Jensen, Bericht über die 48. Tagung für Bäckerei-Technologie und 3. Tagung für Konditorci-Technologie 1997, Vorträge, gehalten anlässlich der

Eric Potter Clarkson  
for opponent Novozymes A/S

Tagung der Arbeitsgemeinschaft Getreideforschung e.V. vom 4. - 6. November 1997 - - Detmold: Granum-Verl., 1998. 167 S. : Ill., graph. Darst. - (Veröffentlichungen der Arbeitsgemeinschaft Getreideforschung e.V. ; 274)

D12 JP-A-55-131340

D12A Abstract of D12 (in English)

D13 GB 1086550 (R T Vanderbilt Company, Inc)

D14 Marion *et al* in Wheat Structure, Biochemistry and Functionality, "Wheat Lipids and Lipid-Binding Proteins: Structure and Function", The Royal Society of Chemistry, Proceedings of a conference organised by the Royal Society of Chemistry Food Chemistry Group, held on 10-12 April 1995, Reading, UK

D15 Marion *et al*, Interactions: The Keys to Cereal Quality, published by the American Society of Cereal Chemists on March 4, 1998

D15A Letter from the publishers of D15 confirming the publication date

D16 WO 03/099016 (Danisco A/S)

A1 Danisco A/S's notice of opposition to EP 0575133

E1 Experimental report on baking performance of prior art lipases

E2 Experimental report on stability and activity of prior art lipases

D1 is cited to show that the patent contains added subject matter.

D2 is cited to show the scope of the claims in the parent patent.

D3 is cited to show that the patent is not entitled to the priority date.

D4, D5, D8, D9, D10, D11, D12 and D13 are cited to show that lipases used in baking had all of the features of Claim 1 in the patent.

D6 is cited to show that, according to the proprietor, SP972 has the hydrolysing and baking properties of Claim 1 in the patent.

D7 is provided to confirm that SP972 is the lipase according to D5.

Eric Potter Clarkson  
for opponent Novozymes A/S

26

D10, D14 and D15 are cited to show that it was known to use lipases in baking to improve pore homogeneity and reduce pore diameter by hydrolysis of digalactosyl diglycerides to the corresponding monoglycerides.

D15A is evidence of the publication date of D15.

D16 shows that Grindamyl EXEL 16 is the lipase of D4 from *Humicola lanuginosa*.

A1 is cited to show the proprietor's view on insufficiency.

E1 shows that the effects of the lipases of the prior art in baking are the same as those specified in Claim 1.

E2 shows that the lipases of the prior art have the stability and fatty acyl hydrolysing features of Claim 1.



I E Stevens  
Eric Potter Clarkson  
July 2005

Eric Potter Clarkson  
for opponent Novozymes A/S

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ BLACK BORDERS
- ☒ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☒ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**